

**CELLULAR VACCINES AND IMMUNOTHERAPEUTICS
AND METHODS FOR THEIR PREPARATION**

RELATED APPLICATIONS

5 The present application is a continuation-in-part of Application Serial No. 08/872,527, filed June 11, 1997, which claims the priority benefit of U.S. provisional application 60/019,639, filed June 12, 1996, all of which are incorporated by reference herein.

FIELD OF THE INVENTION

10 The present invention provides a method for enhancing the immunogenicity of weakly immunogenic or non-immunogenic cells in order to provide the immune system with an immunogenic signal capable of stimulating T cell activation leading to an effective immune response. The method of the invention generates cellular vaccines which are useful for the prevention and treatment of diseases which develop and/or persist by escaping the immune 15 response triggered by T cell activation. Such diseases include, for example, all cancers, natural and induced immune-deficiency states, and diseases caused by infections with a variety of pathogens.

BACKGROUND OF THE INVENTION

20 U.S. patent 5,484,596 by Hanna et al. describes using tumor tissue as a vaccine. U.S. Patent 4,844,893 by Honsik et al. describes arming IL-2-activated leukocytes with Mabs directed to antigens preferentially expressed on tumor cells for killing the target cells. Both patents are incorporated by reference herein.

25 Anti-tumor immune responses are mediated primarily by T lymphocytes. Down regulation of both the major histocompatibility complex (MHC) and the molecules that costimulate the immune response is associated with defective T cells activation signaling by tumor cells (Luboldt et al., *Cancer Res.* 56:826-830, 1996; L. Chen et al., 1992, *Cell* 1: 1093; P.S. Linsley, J. A. Ledbetter, 1993, *Ann. Rev. Immunol.* 11: 191; G.J. Freeman et al., 1993, *Science* 262: 909; C.H. June et al., 1994, *Immune. Today* 15: 321; J. T. Gerge et al., 1993, *Cancer Res.* 53: 2374; Ostrand-Rosenberg, 1993, *S. Curr. Opin. Immune.* 6: 772; B. E. Elliot et al., 1989, *Adv. Cancer Res.* 53: 181).

T cell receptor (TCR) recognition of MHC-bound antigen is not a sufficient signal for T cell activation. Costimulatory molecules, such as B7-1 and B7-2, are cell surface proteins of antigen presenting cells (APCs), and other cells targeted by the immune response, that provide critical signals for T cell activation (for review, see L. Chen et al., 1995, *Immunol*.

5 Rev. 145: 123; T. Tykocinski et al., 1996, *Am. J. Path.* 148: 1). B7 signaling via the T cell surface molecule CD28 appears to be the major costimulatory pathway for T cell activation. However, recent studies show that costimulation is a more complex event which involves both cytokines and adhesion molecules (G. Yang et al., 1995, *J. Immune*. 154: 2794; M. Kubin et al., 1994, *J. Exp. Med.* 180: 211; Y. Li et al., 1996, *J. Exp. Med.* 183: 639).

10 Many approaches have been used to enhance the immunogenicity of tumor cells (see, for example, the references cited in this section). The major approaches presently under investigation involve gene transfer. In this regard, most of the methods employed to date have required *ex vivo* or *in vivo* transfection with genes such as MHC or B7, or modification of tumor cells with antigen presenting cells (APCs) (Y. J. Guo et al., 1994, *Science* 263: 518; M. Tykocinski, 1996, *A. J. Path.* 148: 1; J. Young and K. Inaba, 1996, *J. Exp. Med.* 183: 7; L. Zitvogel et al., 1996, *J. Exp. Med.* 183: 87; C. M. Celluzzi et al., 1996, *J. Exp. Med.* 183: 283). These approaches are time consuming and problematic because of the poor transfectability of primary tumor cells and because of the requirement for large numbers of APCs.

20 *In vitro* treatment of tumor cells with cytokines increases the expression of MHC and adhesion molecules (R. Mattsson et al., 1992, *Biol-Reprod* 46: 1176; R. J. Ulevitch et al., 1991, *Am. J. Pathol.* 139: 287; F. Willems et al., 1994, *Eur. J. Immune*. 24: 1007; I. Saito et al., 1993, *J. Clin. Lab. Anal.* 7: 180; R. A. Panettieri et al., 1994, *J. Immune*. 154: 1358; M. Ikeda et al., 1994, *J. Invest. Dermatol.* 103: 791). Transfection of tumor cells with MHC, B7-25 1 and B7-2 genes converts low immunogenic tumor cell lines to immunogenic cell lines (S. E. Townsend and J. P. Allison, *Science* 259: 368; J. P. Allison et al., 1995, *Curr. Opin. Immune*. 7: 682; G. Yang et al., 1995, *J. Immune*. 154: 2794; M. Kubin et al., 1994, *J. Exp. Med.* 180: 211). Non-immunogenic tumor cells are not responsive to transfection with the B7 gene alone but can become responsive by co-expression of CD48 molecules at the cell 30 surface (Y. Li et al., 1996, *J. Exp. Med.* 183: 639).

The costimulatory molecule B7 can under some circumstances deliver a negative

signal through its binding to CTLA-4, a second receptor for B7 on T cells. Cross-linking CTLA-4 molecules *in vitro* has been shown to inhibit T cell proliferation. Furthermore, mice deficient in CTLA-4 develop severe T cell proliferative disorders (K. Kawai et al., 1993, *Science* 261: 609; J. P. Allison, M. K. Krummel, 1995, *Science* 270: 932; J.M. Green et al., 5 1994, *Immunity* 1: 501). A recent report showed that the introduction of anti-CTLA-4 monoclonal antibody (MAb), which blocks CTLA-4 mediated signaling, resulted in enhanced T cell- dependent rejection of tumors in certain mouse models (D.R. Leach et al., 1996, *Science* 271: 1734). These data provide evidence that CTLA-4 may be counter-regulatory to the CD28 costimulatory signal. Thus, transfected tumor cells expressing B7 molecules may 10 fail to elicit effective immunity due to CTLA-4 mediated negative signaling.

In addition to T cell activation using B7 gene transfection, bispecific monoclonal antibodies (Bi-MAbs) in combination with pre-stimulated lymphocytes have been used to induce T cell activation under certain circumstances. For example, one study reports that costimulatory signals can be delivered by a combination of Bi-MAbs to CD28:CD30 (CD30 15 is a Hodgkin's tumor- associated antigen) and CD3:CD30 in combination with peripheral blood lymphocytes (PBLs) pre-stimulated with the CD3:CD30 Bi-MAb in the presence of CD30⁺ Hodgkin's tumor-derived cells; however, the combination of CD28:CD30 and CD3:CD30 Bi-MAbs alone did not induce significant *in vitro* cytotoxicity of resting human PBLs against a Hodgkin's tumor-derived cell line, and stimulation with the CD28:CD30 Bi- 20 MAb alone was not effective (C. Renner et al., 1994, *Science* 264: 833). Similarly, regression of Hodgkin's derived tumor xenografts was observed only when both the CD28:CD30 and CD3:CD30 Bi-MAbs were used in combination with PBLs prestimulated *in vitro* with CD30⁺ cells and CD3:CD30 Bi-MAb; no significant effect was observed in 25 xenografts treated with either of the Bi-MAbs alone, or a combination of the two Bi-MAbs without prestimulated human PBLs (Renner et al., *supra*).

SUMMARY OF THE INVENTION

The present invention features immunogenic tumor cells and other immunogenic cells, methods of making such immunogenic cells, methods of using such immunogenic cells 30 to activate or enhance immune response against diseased cells with minimum effect on normal or healthy cells, and methods of avoiding negative T cell signaling pathways.

The present invention provides a method for enhancing the immunogenicity of cells and especially weakly-immunogenic or non-immunogenic cells, resulting in a cellular vaccine that can stimulate T cell activation *in vivo* or *in vitro*, which in turn leads to an effective immune response against diseased cells. The cellular vaccines of the present invention can be used as vaccines to prevent diseases and as immunotherapeutics to treat diseases. The starting materials for the cellular vaccine can be a target diseased cell, or an antigen presenting cell presenting one or more antigens associated with a disease (e.g., dendritic cells, macrophages, B cells, and other cells fused with diseased cell, pulsed with antigens or transfected with antigen expressing nucleic acid).

In summary, the method of the invention involves the steps of (1) treating target cells (especially weakly- or non-immunogenic cells) in order to amplify primary and costimulatory T cell activation signals in the cells, and (2) attaching to the treated cells a substance capable of binding to one or more antigens on the treated cells and to one or more T cell activation costimulatory molecules on the surface of T cells (such as CD28), thereby providing the treated cells with the capacity to physically link to T cells and to activate the costimulatory signal. Such substances include, but are not limited to, bispecific monoclonal antibodies (Bi-MAbs) targeted to antigen on the treated cells and to CD28 and/or other costimulatory molecules on T cells. The first step may be skipped when the target cell is attached with (1) a bridge molecule with two or more binding sites for T cell activation costimulatory molecules on the surface of T cells, or (2) two or more bridge molecules each with one or more binding sites for T cell activation costimulatory molecules on the surface of T cells. The first step may also be skipped when the target cells are antigen presenting cells presenting one or more antigens associated with a disease.

Once the primary and/or costimulatory T cell activation signals in the target cells have been amplified by cytokines or other means and the bridge molecules have been attached to the target cells, the cytokines and the bridge molecules not attached to the target cells may be removed from the immunogenic composition before the target cells are administered to a patient. This additional step minimizes adverse effects associated with administering cytokines to a patient. It also minimizes the risk associated with allowing bridge molecules not attached to a target cell into a patient, an event which may cause unwanted immune response against normal or healthy cells.

The first step of the method up-regulates antigen processing capacity within the treated cells and amplifies the expression of cell surface molecules involved in T cell activation. The second step provides the treated cells with a means to physically bridge to T cells via CD28 and/or other costimulatory molecules, thereby providing optimal conditions 5 for stimulating T cell activation.

Thus, in a first aspect, this invention features an immunogenic composition for administration to a patient mammal (including a human) having target diseased cells. The immunogenic composition contains a target diseased cell (including, but not limited to, an autologous target diseased cell) which differs from the corresponding diseased cells in the 10 patient in that it processes and presents antigens characteristic of the diseased cells more effectively. For example, the autologous target diseased cell expresses one or more primary (e.g., MHC) and/or costimulatory (e.g., B7-1 and B7-2) T cell activation molecules at a higher level (e.g., 50% higher, preferably 2 folds higher, more preferably 10 folds higher). As described below, there are different ways of enhancing the expression level of the primary 15 and/or costimulatory T cell activation molecules.

In addition, the target diseased cell has attached thereto one or more bridge molecules. Each bridge molecule has one or more binding sites for one or more costimulatory molecules on the surface of effector cells, which include, but are not limited to, T cells, NK cells, macrophages, LAK cells, B cells, and other white blood cells. Preferably, though not required, the bridge molecules have one or more binding sites for one or more antigens on the 20 surface of the target diseased cell and are attached to the target diseased cells at the cell surface antigens. In another preferred embodiment, substantially all (e.g., >80%, preferably >90%, more preferably >95%) the bridge molecules in the immunogenic composition are attached to the autologous target diseased cells so that the composition is substantially free of bridge molecules not attached to a target diseased cell. In a further preferred embodiment, the 25 immunogenic composition contains a pharmaceutically effective amount of the target diseased cells with bridge molecules attached thereto.

By "immunogenic" is meant the ability to activate the response of the whole or part of the immune system of a mammal, especially the response of T cells, and more especially the 30 response of CD8⁺ T cells.

By "target diseased cell" is meant (a) a cell causing, propagating, aggravating or

contributing to a disease in a patient mammal, (b) a cell capable of causing, propagating, aggravating or contributing to a disease in a patient mammal, (c) a cell derived from (a) or (b), or (d) a cell sharing antigenic characteristics of (a) or (b). Target diseased cells may or may not be taken from the patient mammal to be treated by a method of this invention.

5 Target diseased cells include, but are not limited to, tumor cells (including unmodified tumor cells, tumor cells modified with different approaches, primary cultures of tumor cells, and established cancer cell lines). The sources of tumor cells include, but are not limited to, liver cancer, hepatocellular carcinoma, lung cancer, gastric cancer, colorectal carcinoma, renal carcinoma, head and neck cancers, sarcoma, lymphoma, leukemia, brain tumors,

10 10 sterosarcoma, bladder carcinoma, myloma, melanoma, breast cancer, prostate cancer, ovarian cancer, and pancreas carcinoma. In addition, target diseased cells include cells transformed or immortalized by irradiation, viral infection, exposure to carcinogens, and other means known to or to be developed by those skilled in the art. Target diseased cells may also be cells infected with prions (which cause Mad Cow diseases among others),

15 15 viruses, bacteria, fungi, protozoa or other parasites (e.g. worms).

Viruses include those described or referred to in Fields Virology, Second Edition, 1990, Raven Press, New York, incorporated by reference herein. Examples include, but are not limited to, herpes virus, rhinoviruses, hepatitis virus (type A, B, C and D), HIV, EBV, HPV, and HLV.

20 20 Bacteria include those described or referred to in Bergey's Manual of Determinative Bacteriology, Ninth Edition, 1994, Williams and Wilkins, incorporated by reference herein. Examples include, but are not limited to, gram positive and negative bacteria, *streptococci*, *pseudomonas* and *enterococci*, *Mycobacterium tuberculosis*, *Aeromonas hydrophilia*, *Aeromonas caviae*, *Aeromonas sobria*, *Streptococcus uberis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Bacillus sphaericus*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Serratia liquefaciens*, *Lactococcus lactis*, *Xanthomonas maltophilia*, *Staphylococcus simulans*, *Staphylococcus hominis*, *Streptococcus constellatus*, *Streptococcus anginosus*, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium fortuitum*, and *Klebsiella pneumonia*.

30 30 By "autologous" is meant that the target diseased cell is from the patient mammal, or from another patient having a common major histocompatibility phenotype. An autologous

target cell may be obtained from the patient mammal or another source sharing the same MHC with methods known to those skilled in the art. Once taken from a patient, an autologous cell may be modified, transfected, and treated by methods described herein and other methods known to or to be developed by those skilled in the art..

5 Primary T cell activation molecules include MHC class I, MHC class II and other molecules associated with antigen processing and/or presentation. Costimulatory T cell activation molecules include ICAM-1, ICAM-2, ICAM-3, LFA-1, LFA-2, VLA-1, VCAM-1, 4-1-BB, B7-1, B7-2, and other cell adhesion proteins and other cell surface proteins which can activate T cell costimulatory pathways through T cell surface proteins.

10 By "bridge molecule" is meant a molecule or substance which can bring two or more cells together by attaching to the cells with its binding sites. Preferably, a bridge molecule can bring an autologous target diseased cell together with an effector cell and deliver a signal to the effector cell to activate or enhance the effector cell's immune response against the target. A bridge molecule has one or more binding sites for stimulatory and/or costimulatory 15 molecules on the effector cells. These binding sites can be designed to activate a positive regulator of T cell activation (e.g., CD28, 4-1BB) but avoid stimulating a negative regulator of T cell activation (e.g., CTLA-4). The binding sites can also be designed to blockade a negative regulator of T cell activation (see Leach et al., Science 271:1734-1736, 1996). A bridge molecule may also have one or more binding sites for antigens on the surface of the 20 target diseased cell. Bridge molecules include, but are not limited to, bispecific monoclonal antibodies, fusion proteins, organic polymers, and hybrids of chemical and biochemical materials. The antibodies described or disclosed in U.S. patents 5,601,819, 5,637,481, 5,635,602, 5,635,600, 5,591,828, 5,292,668 and 5,582,996 are incorporated by reference herein.

25 The antigen on the target cell serving as an anchor for the bridge molecule need not be unique to the target cell when the bridge molecule is attached to the target cell *in vitro*. Any molecule on the target cell surface can be used to anchor the bridge molecule, including, but not limited to, proteins, glycoproteins, lipids, glycolipids, phospholipids, lipid aggregates, steroids, and carbohydrate groups such as disaccharides, oligosaccharides and 30 polysaccharides (see "Molecular Biology of The Cell," pp47-58, pp276-337, Second Edition, published by Garland Publishing, Inc. NY & London). Examples include transferrin receptor,

Low Density Lipoprotein (LDL) receptor, gp55, gp95, gp115, gp210, CD44, ICAM-1, ICAM-2, collagen and fibronectin receptor, transferrin receptors, Fc receptor, and cytokine receptors.

Costimulatory molecules on the surface of effector cells may be antigens, fatty acids,

- 5 lipids, steroids and sugars that can stimulate or costimulate these effector cells' functions to destroy the target cells. Costimulatory molecules include, but are not limited to, CD1a, CD1b, CD1c, CD2, CD2R, CD3, CD4, CD5, CD6, CD7, CD8, CD9, CD10, CD11a, CD11b, CD11c, CDw12, CD13, CD14, CD15, CD15s, CD16a, CD16b, CDw17, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD30, CD31, CD32, 10 CD33, CD34, CD35, CD36, CD37, CD38, CD39, CD40, CD41, CD42a, CD42b, CD42c, CD42d, CD43, CD44, CD44R, CD45, CD45RA, CD45RB, CD45RO, CD46, CD47, CD48, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD50, CD51, CD51/61 complex, CD52, CD53, CD54, CD55, CD56, CD57, CD58, CD59, CDw60, CD61, CD62E, CD62L, CD62P, CD63, CD64, CDw65, CD66a, CD66b, CD66c, CD66d, CD66e, CD67, CD68, CD69, CD70, 15 CD71, CD72, CD73, CD74, CDw75, CDw76, CD77, CDw78, CD79a, CD80, CD81, CD82, CD83, CDw84, CD85, CD86, CD87, CD88, CD89, CDw90, CD91, CDw92, CD93, CD94, CD95, CD96, CD97, CD98, CD99, CD99R, CD100, CDw101, CD102, CD103, CD104, CD105, CD106, CD107a, CD107b, CDw108, CDw109, CD110-CD114, CD115, CDw116, CD117, CD118*, CD119, CD120a, CD120b, CDw121a, CDw121b, CD122, CD123*, 20 CDw124, CD125*, CD126, CDw127, CDw128, CD129, CDw130, LFA-1, LFA-2, LFA-3, VLA-1, VCAM-1, VCAM-2, 4-1BB, cytokine and chemokin receptors. In a preferred embodiment, the bridge molecule has a binding site for CD28 or 4-1BB on the surface of T cells.

By "pharmaceutically effective" is meant the ability to cure, reduce or prevent one or

- 25 more clinical symptoms caused by or associated with the diseased cells in the patient mammal, including, but not limited to, uncontrolled cell proliferation, bacteria infection, and virus infection.

The immunogenic composition may be isolated, enriched or purified for administration to a patient.

- 30 By "isolated" in reference to the immunogenic composition is meant that the autologous target diseased cell is isolated from a natural source. Use of the term "isolated"

indicates that one or more naturally occurring materials have been removed from the normal environment. Thus, the target diseased cell may be placed in a different cellular environment or in a solution free of other cells. The term does not imply that the target diseased cell is the only cell present, but does indicate that it is the predominate cell present (at least 20 - 50% more than any other cells) and is essentially free (about 90 % pure at least) of other tissues naturally associated with it in the body of the patient. In a preferred embodiment, the composition is substantially free of effector cells such as T cells. In another preferred embodiment, the composition is substantially free of bridge molecules not attached to a target diseased cell. In a third preferred embodiment, the composition is substantially free of cytokines outside of the target diseased cell.

By "enriched" in reference to the immunogenic composition is meant that the autologous target diseased cell constitutes a significantly higher fraction (2 - 5 fold) of the total cells in the composition than in the diseased tissue in the patient's body. This could be caused by a person by preferential reduction in the amount of other cells present, or by a preferential increase in the amount of the specific target diseased cells, or by a combination of the two. However, it should be noted that enriched does not imply that there are no other cells present, just that the relative amount of the cell of interest has been significantly increased in a useful manner. The term "significantly" here is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other cells of about at least 2 fold, more preferably at least 5 to 10 fold or even more.

By "purified" in reference to the immunogenic composition does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the target diseased cell is relatively purer than in the natural environment. The target diseased cells could be obtained directly from the patient or from cell culture, with or without modifications. Purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. In a preferred embodiment, the composition is substantially free of effector cells such as T cells.

The immunogenic composition may contain a pharmaceutically suitable carrier or excipient. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990). The

immunogenic composition may be administered to a patient systemically, e.g., by intravenous infusion or subcutaneous injection. A composition of the invention may be administered as a unit dose to a patient mammal, each unit containing a predetermined quantity (e.g., about 1x10⁵ to about 1x10¹⁰, preferably about 1x10⁶ to about 1x10⁹, and more preferably about 5 1x10⁷ to about 1x10⁸) of armed and/or activated autologous target diseased cells calculated to produce the desired therapeutic effect in association with the physiologically tolerable aqueous medium as diluent.

The expression of primary and costimulatory T cell activation molecules may be enhanced by various means, for example, *in vitro*, *ex vivo* or *in vivo* treatment of target cells 10 with cytokines or other factors capable of inducing the desired amplification; and *in vitro* and *in vivo* transfer to the target cells of MHC genes, adhesion molecule genes, cytokine genes, and/or their respective transcription activators or enhancers. Cytokines include those described or referred to in The Cytokine Handbook, Thomson, A., (ed.), 1994, Academic Press, San Diego, incorporated by reference herein. Examples include, but are not limited to, 15 IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, G-CSF, GM-CSF, interferons (e.g., IFN α , β , and γ), tumor necrosis factors (e.g., TNF α , and β) and other chemokines and lymphokines. In a preferred embodiment, IFN γ and TNF α are used either alone or in combination to enhance the expression of primary and costimulatory T cell activation molecules in autologous target diseased cells.

20 The bridge molecule may be attached to the target cells by various means, for example, *in vitro*, *ex vivo* or *in vivo* treatment of target cells with the bridge molecule. When a target diseased cell coated with bridge molecules is administered into a patient, it will bind 25 to costimulatory molecules on the surface of the effector cells. The more densely the target diseased cell is coated with bridge molecules, the more effector cells it will be able to bind. In addition, the more binding sites a bridge molecule has for the costimulatory molecules, the more effector cells it will be able to bind.

In that regard, Applicant has found that a cellular vaccine may be prepared without the 30 need of cytokine treatment (to increase the levels of primary and costimulatory T cell activation molecules) when a plurality of bridge molecules are attached to a target cell with binding sites for two or more different costimulatory molecules on the surface of T cells (e.g., CD3, CD28, and 4-1BB). Individual bridge molecules may be attached to different anchor

molecules on the surface of the target diseased cell. An individual bridge molecule may also have two or more binding sites for two or more different costimulatory molecules on the surface of T cells.

Thus, in a second aspect, this invention features an immunogenic composition
5 containing an autologous target diseased cell having attached thereto (a) a bridge molecule which has two or more binding sites for two or more different effector cells, (b) a bridge molecule which has two or more binding sites for two or more different costimulatory molecules on the surface of effector cells, (c) two or more bridge molecules each containing a binding site for a different effector cell, (d) two or more bridge molecules each containing a
10 binding site for a different costimulatory molecule on the surface of effector cells, (d) two or more bridge molecules each attached to a different antigen on the target cells, or (e) a combination of two or more of the above.

A pharmaceutically effective amount of an immunogenic composition of this invention may be complemented by a pharmaceutically acceptable carrier before
15 administration to a patient mammal.

Alternatively, a patient may be administered with a pharmaceutical composition containing (1) a pharmaceutically effective amount of a cytokine capable of increasing the level of one or more primary and costimulatory T cell activation molecules in tumor cells, (2) a pharmaceutically effective amount of a bridge molecule containing a binding site for an
20 antigen on the surface of the tumor cells and a binding site for a costimulatory molecules on the surface of T cells, and (3) a pharmaceutically acceptable carrier. The administration may be systemic or local.

In treating a patient, the target cell may be treated with cytokines or other means of increasing primary and costimulatory T cell activation molecules *in vitro* before the target
25 cell is administered to the patient. Alternatively, the cytokines may be administered to the patient to increase primary and costimulatory T cell activation molecules *in vivo*.

In a third aspect, this invention features a method of generating cytotoxic leukocytes against diseased cells in a patient mammal by contacting a population of effector cells (e.g., white blood cells) *in vitro* with immunogenic compositions described above for a time period
30 sufficient to react with the immunogenic compositions and collecting the treated effector cell population. The cytotoxic leukocytes so generated can then be administered to a patient to

treat or prevent diseases. This adoptive immunotherapy can be used alone or in combination with vaccination to treat or prevent diseases.

The method of the invention is useful for the prevention and treatment of diseases which develop and/or persist by escaping immune responses triggered by T cell activation.

5 Such diseases include, for example, all cancers, natural and induced immune deficiency states, and diseases caused by infections with a variety of pathogens. The method of the invention is illustrated herein by demonstrating its application to three different types of human cancers. Cancer cells are by nature generally weakly immunogenic, fail to trigger an effective T cell response, and survive and grow as a result. As demonstrated herein, cancers 10 can be prevented, and established cancers may be cured, by stimulating an effective T cell response using autologous tumor cell vaccines of the invention.

Other features and advantages of the invention will be apparent from the following detailed description of the invention, and from the claims.

15 **BRIEF DESCRIPTION OF THE FIGURES**

FIG. 1. Expression of MHC class I, ICAM-1, ICAM-2 and VCAM-1 antigens on cytokine treated hepa 1-6 cells. The results are representative data from four comparable experiments.

20 FIG. 2. Stimulation and proliferation of syngeneic splenic T cells *in vitro* induced by cytokine treated hepa 1-6 cells and anti-CD28 MAb.

FIG. 3. Cytotoxicity of CTLs generated by *in vitro* priming of naive splenic T cells with cytokine treated hepa 1-6 cells in combination with anti-CD28 Bi-MAbs or control MAb.

25 FIG. 4. Induction of protective immunity with cytokine treated hepa 1-6 cells armed with anti-CD28 Bi-MAb.

FIG. 5. Tumor rejection following treatment with cytokine treated hepa 1-6 cells armed with CD28:gp55 Bi-MAb.

FIG. 6. Cure of established hepatomas *in vivo*.

30 FIG. 7. Therapeutic effectiveness of γ -irradiated cytokine treated hepa 1-6 cells armed with CD28:gp55 Bi-MAb.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for immunizing individuals against disease and for treating individuals with established diseases using cellular vaccines created with a two-step process described herein. The methods of the invention may be applicable to any disorder involving a low- or non-immunogenic response pathology, wherein effective treatment or prophylaxis requires an immune boost through activation of T cells. Such disorders include, but are not limited to, all forms of cancer, immune deficiency disorders (both natural and induced), and infectious diseases caused by viral or other pathogenic agents.

10 A. Current Approaches to the Generation of Cellular Vaccines Against Tumors

Anti-tumor immune responses are primarily mediated by T cells. For optimal activation of T cells, at least two signals are essential. The first is an antigen-specific signal via the T cells receptor and MHC-polypeptide complex interaction. The second is a costimulatory signal mediated via a different set of receptor / counter-receptor pathway. 15 Down-regulation expression of MHC and the molecules that costimulate the immune responses are associated with defective signaling of tumor cells for T cell activation. The immunogenic potential of tumor cells can be enhanced through defined molecular modifications. The modified tumor cells can be used as cellular vaccines to elicit anti-tumor specific immunity that is effective in eradicating established tumors in animals. Most of 20 these approaches have required *ex vivo* or *in vivo* gene transfection with a viral vector or modification of tumor cells with antigen processing cells (APCs).

1). Gene transfer to tumor cells

Many approaches have been used to enhance immunogenicity of tumor cells. In early 25 studies, the stimulatory signals were provided exogenously by immunizing animals with tumor cells mixed with adjuvants or with chemically modified tumor cells. Recent advances in genetic engineering allow the modification of tumor cells by gene transfection. For example, transfection of MHC genes into tumor cells converted non-immunogenic tumor cells into immunogenic ones (Haddada et al., Proc Natl Acad Sci USA 1986, 83:9684-9688; 30 and Wallich et al., Nature 1985, 315:301-315). Immunization of animals with tumor cells transfected with cytokine genes, including IL-1 (Ziller et al., Intl J Cancer 1992, 50:450-

457), IL-2 (Bubenik et al., Immunol Lett 1990, 23:287-292), IL-4 (Tepper et al., Cell 1989, 57:503-512), IL-6 (Porgador et al., Cancer Res 1992, 52:3679-3683), IL-7 (Hock et al., J Exp Med 1991, 174:1291-1298), TNF (Asher et al., J Immunol 1991, 146:3227-3235), G-CSF (Colombo et al., J Exp Med 1991, 173:889-897), GM-CSF (Dranoff et al., Proc Natl Acad Sci USA 1993, 90:3539-3543), IP-10 (Luster et al., J Exp Med 1993, 178:1057-1065) and IFN γ (Gansbacher et al., Cancer Res 1990, 50:7820-7825) also induced host anti-tumor immune responses. In some of these studies, anti-tumor immunity was mediated by T lymphocytes. In other studies, anti-tumor immunity was mediated by macrophages or neutrophils. Inhibition of insulin like growth factor production by transfection with a vector expressing anti-sense 5 IGF-I rendered rodent C6 glioma more immunogenic (Trojan et al., Science 1993, 259:94-10 96).

Tumor cells (J558L) were engineered to over-produce one of 5 different cytokines (IL-2, IL-4, IL-7, TNF, or IFN γ (Hock et al., Cancer Res 1993, 53:714-716). These cytokine producing tumor cells were then used to immunize animals against a challenge with parental 15 tumor cells. Injection of all cytokine producing tumor cells induced systemic responses capable of mediating the rejection of parental tumor cells when injected in low numbers.

Gene transfection approaches usually require the establishment of cells in tissue culture and transfection of primary tumor cells and selection *in vitro*. They are time-consuming and costly. The clinical application may thus be limited.

To circumvent requirements for the establishment of tumor cell lines *in vitro*, the direct introduction of genes into tumor cells *in situ* has been attempted. A gene encoding an allogeneic HLA antigen was incorporated into liposome, and injected into five patients with advanced melanoma. One patient demonstrated regression of tumor nodules. In this patient, metastatic lesions at distant sites also displayed complete regression. The other four patients 20 did not respond to the treatment. These results suggest that introduction of exogenous genes directly into tumors has potential as a therapeutic approach (Nabel et al., Ann. NY Acad Sci 1995, 27:227-231).

2). Antigen presentation by APCs

An effective tumor vaccine should have all the signals essential for T cell activation. Activated B cells are very effective antigen-presenting cells. In addition to expressing of high 30

levels of MHC class II antigens, activated B cells express high levels of accessory and costimulatory molecules. In one approach, BERH-2 cells, a chemically induced rat hepatoma cell line, were fused with *in vivo* activated B cells. The hybrid tumor cells expressed high levels of MHC class I, II, ICAM-1 and B7 and lost tumorigenicity in syngeneic animals.

5 Animals immunized with the hybrid cells generated by fusion of tumor cells with either activated B cells or dendritic cells became resistant to parental tumor challenge and cured established tumors (Toffaletti et al., *J Immunol* 1983, 130:2982-2986; and Guo et al., *Science* 1994, 263:518-520).

Dendritic cells transfected with tumor antigen expressing nucleic acids were able to be 10 used as tumor cellular vaccines to stimulate T cell immunity (Liu, 1998, *Nature Biotechnology* 16:335). Several recent papers have showed that autologous dendritic cells pulsed *ex vivo* with tumor antigens including tumor associated antigen or tumor specific 15 idiotype protein were able to be used as tumor cellular vaccines to stimulate host immunity (Young et al., *J. Exp. Med.* 1996, 183:7-11; Celluzzi et al., *J. Exp. Med.* 1996, 183:283-287; Hsu et al., *Nature Med.* 1996, 2(1): 52-58; and Gong et al., *Nature Med.* 1997, 3(5): 558- 20 561). In contrast to gene transfection experiments in which only one or two genes can be introduced into tumor cells at a time, fusion of tumor cells with APCs or modification of dendritic cells with tumor antigens *ex vivo* gives rise to treated APCs with both tumor specific and costimulatory signals. However, these approaches are still time-consuming and requiring large numbers of activated B cell and dendritic cells.

B. Improvement and Alternative to the Current Approaches

The present invention provides improvements and alternatives to the current approaches to generate cellular vaccines against cancer and other diseases.

25 In one embodiment, the methods of the invention comprise modifying antigen-presenting cells (APCs) by (1) treating the APCs in order to present on the cell surface thereof antigens derived from or associated with the target diseased cells, and (2) attaching to the APCs a bridge molecule capable of binding to one or more T cell activation costimulatory molecules on the surface of T cells (e.g., CD28), thereby providing the APCs with the 30 capacity to physically link to T cells and activate the costimulatory signal. For the first step, the APCs may be (a) transfected with foreign nucleic acid capable of expressing within the

APCs antigens derived from or associated with the target diseased cells (e.g., Donnelly *et al.*, 1994, J. Immunol. Methods 176:145), (b) pulsed with tumor lysate (e.g., Nestle *et al.*, 1998, Nat. Med. 4:238), or (c) pulsed with purified tumor associated peptides or proteins (e.g., Mayordomo *et al.*, 1995, Nat. Med. 1:1297; Porgador *et al.*, 1995, J. Exp. Med. 182:255; 5 Celluzzi *et al.*, 1996, J. Exp. Med. 183:283; Paglia *et al.*, 1996, J. Exp. Med. 183:317; Bakker *et al.*, 1995, Cancer Res. 55:5330; Hsu *et al.*, 1996, Nat. Med. 2:52; Reeves *et al.*, 1996, Cancer Res. 56:5672; Zitvogel *et al.*, 1996, J. Exp. Med. 183:87; and Boczkowski *et al.*, 1996, J. Exp. Med. 184:465).

In another embodiment, the methods of the invention comprise modifying target cells 10 (including, but not limited to, weakly- or non-immunogenic autologous cells of the disorder) by (1) treating the target cells in order to amplify primary and costimulatory T cell activation signals therein, and (2) attaching to the target cells a substance capable of binding to one or more antigens on the target cells and to one or more T cell activation costimulatory molecules on the surface of T cells (e.g., CD28), thereby providing the target cells with the capacity to 15 physically link to T cells and activate the costimulatory signal. Such substances include, but are not limited to, bispecific monoclonal antibodies (Bi-MAbs) targeted to antigen on the treated cells and to CD28 and/or other costimulatory molecules on T cells.

The first step of the method amplifies the expression of cell surface molecules 20 involved in T cell activation, such as MHC and adhesion molecules, and up-regulates antigen processing capacity within the target cells by enhancing enzyme activity involved in intracellular antigen processing. For the first step of the method, any means which can amplify primary and costimulatory T cell activation signals in the target cells (e.g., the expression of MHC and adhesion molecules), may be used. Such amplified expression may be achieved by, for example, *in vitro* and *in vivo* treatment of target cells with cytokines or 25 other factors capable of inducing the desired amplification; and *in vitro* and *in vivo* transfer of MHC genes, adhesion molecule genes, cytokine genes, and/or MHC, adhesion molecule, and cytokine gene transcription activators or enhancers to the target cells. Specific examples include (a) introduction of gene encoding MHC Ags (Restifo *et al.*, 1993, J. Immunother. 14:182; Ostrand-Rosenberg *et al.*, 1990, J. Immunol. 144:4068; and Armstrong *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:6886), costimulatory molecule (Chen *et al.*, 1992, Cell 30 71:1093; Townsend *et al.*, 1993, Science 259:368; Baskar *et al.*, 1993, Proc. Natl. Acad. Sci.

USA 90:5687; and Johnston *et al.*, 1996, J. Exp. Med. 183:791), or cytokines (Fearon *et al.*, 1990, Cell 60:397; Golumbek *et al.*, 1991, Science 254:713; and Dranoff *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:3539) into tumor cells; (b) fusion of tumor cells with antigen presenting cells (APCs) (Guo *et al.*, 1994, Science 263:518; WO 95/16775; Gong *et al.*, 1997, 5 Nat. Med. 3:558; and Wang *et al.*, 1998, J. Immunol. 161:5516); and (c) stimulation with virus infection (Von Hoegen, *et al.*, 1990, Cell. Immunol. 126:80; Ockert *et al.*, 1996, Clin. Cancer Res. 2:21; Ertel *et al.*, 1993, Eur. J. Immunol. 23:2592; and Jurianz *et al.*, 1995, Int. J. Oncol. 7:539).

In one embodiment of the method, amplification of primary and costimulatory T cell activation signals in the target cells is achieved using cytokine treatment. Target cells may be treated with cytokines *ex vivo* or *in vitro* as described in the examples herein. Alternatively, cytokines may be administered to the target cells *in vivo* by, for example, intralesional injection, intralymph injection, intravesical injection, subcutaneous injection, etc., in suitable pharmaceutical carriers or controlled release preparations. Any cytokine or combination of 10 cytokines which results in the amplified expression of MHC and adhesion molecules may be used to treat cells in the first step of the method. In preferred embodiments, described more fully by way of the examples herein, a combination of interferon (IFN- γ) and tumor necrosis factor- α (TNF α) is used in the first step. Preferably, cells may be treated with concentrations of between about 10 - 100 U IFN- γ in combination with concentrations of between about 10- 15 100 U TNF α , more preferably with 100 U IFN γ , and 50 U TNF α , as described in Example 1. 20 However, the conditions and specific cytokines most optimal for the amplification of activation signals on the particular cells to be treated may vary and may be determined essentially as described in Example 1.

The second step of the method of the invention provides the treated cells with the 25 capacity to physically bridge to T cell surfaces via CD28 and/or other T cell costimulatory molecules, thereby providing optimal conditions for stimulating T cell activation. For the second step, any substance capable of binding to one or more antigens on the treated cells and to one or more T cell activation costimulatory molecules on the surface of T cells may be used. Such bispecific or multispecific bridging substances may comprise, for example, Bi- 30 Mabs, proteins and other macromolecules, and polymer materials, which contain a functionality capable of binding to the targeted T cell costimulatory molecule and activating,

or inducing the activation of, the costimulatory signal. In one embodiment, described by way of the examples in Example 6, Bi-MAbs are used as the bridging substance.

One functionality of the bispecific or multispecific bridging substance may be directed to a target cell-specific antigen or any antigen expressed on the target cells.

5 Optimally, where the target cells are to be armed with bridging substance *in vivo*, the target cell antigen to which the bridging substance is directed should be unique.

10 However, the target cell antigen need not be unique to the treated cells, since the attachment of the bridging substance may be practiced *in vitro*. Accordingly, bridging substances attached to the target cells *in vitro* will not cross-react with the same antigen on cells in the individual to be immunized with the modified cells.

After such bridging substances are incubated with cells treated according to the first step of the method, free bridging substance may be washed away and bound bridging substance may be cross-linked to the cell surface with polyethylene glycol (PEG) or another cross-linking agent.

15 Another functionality of the bispecific or multispecific bridging substance is specifically directed to a T cell activation costimulator such as CD28. Thus, when the modified cells are used to immunize an individual, the CD28 (or other costimulatory molecule) binding sites of the attached bridging substance are free, and will bind to CD28 (or other costimulatory molecule) on T cell surfaces, ensuring that the modified cells will become 20 physically linked to T cells. This bridging substance-mediated physical link also brings other molecules on the surfaces of the modified cells, some of which have been amplified by the cytokine treatment step, into contact with other molecules on the surfaces of T cells, providing further costimulation which thereby further facilitates T cell activation.

25 The second step of the method may be practiced *in vitro* or *in vivo*, depending upon whether target cells were treated according to the first step of the method *in vivo* or *in vitro*, the circumstances of the disease or lesion to be treated, and the clinical objectives of the treatment. Where the first step of the method is conducted *in vivo*, the treated cells may be 30 armed with the bridging substance *in vivo* as well. In this case, the clinician may use a variety of known methods for administering the bridging substance to a patient. The best route of administering the bridging substance to patients who have had disease- or lesion-specific cells (target cells) treated *in vivo* according to the first step of the method will depend

on clinical and/or other aspects of the disease or lesion to be treated as well as on the site of the treated cells. For example, where target cells located in lymph node have been treated *in vivo*, direct administration of the bridging substance to the lymph node is preferred.

5 Similarly, for example, where tumor cells have been treated *in vivo* by intratumor injection of cytokines or gene transfer vectors, the bridging substance preferably should be administered directly into the tumor or to the local environment of the tumor.

Where the first step of the method is conducted *in vitro*, the treated cells may be armed with the bridging substance *in vivo* or *in vitro*. Where the bridging substance is administered *in vivo*, the same route used to administer the treated cells or a similar route 10 should be used, taking into account the same factors discussed above regarding *in vivo* arming of *in vivo* treated target cells. The second step may be taken at the same time with the first step or at a different time.

When *in vitro* treated cells are armed *in vitro*, the treated and armed cells (cellular vaccine) may be used *in vivo* for treatment and prevention of disease, or *in vitro* for 15 generation of lesion- or disease- specific cytotoxic T lymphocytes (CTLs). Arming treated cells *in vitro* provides the advantage of being able to use a bridging substance directed to any antigen on the target cell.

When used for treatment or immunization of a patient, *in vitro* treated and armed cells may be administered to the patient using a variety of methods known to those skilled in the 20 art. In a particular embodiment, described more fully in the examples which follow, *in vitro* treated and armed cells are administered subcutaneously. In another embodiment, the treated and armed cells are administered by direct intralesion injection, an administration route that may provide advantages over subcutaneous administration in certain circumstances (for example, where the lesion to be treated is not well vascularized, is inaccessible for biopsy, or 25 cannot be disrupted without creating further risk to the patient). In a further embodiment, the treated and armed cells are administered by injection into the lymph nodes. This method of administration requires fewer treated and armed cells than may typically be required using other routes of administration. Such intralymph administration may be preferred in situations where only limited autologous tissue can be obtained from a lesion using thin needle biopsy 30 techniques (e.g., inaccessible/inoperable cancers). Moreover, intralymph administration is likely to enhance the interaction between the cellular vaccine and T cells given the large

number of T cells within lymph nodes. Applicant's initial experimental data indicates that a single intralymph injection of as few as 1 X 10⁴ cellular cancer vaccine cells, prepared using the method of the invention, can induce an effective immune response against parenteral tumor cell challenge and can cure established tumors. The therapeutic efficacy of this method 5 of administration appears equivalent to that achieved using 100-times more cells administered subcutaneously.

In a specific embodiment of the method of the invention, Bi-MAbs that react with CD28 are used as a bispecific bridging substance. B7 interacts with both CD28 and CTLA-4 on T cells. Under certain circumstances, the B7-CTLA-4 interaction generates a negative 10 signal which prevents T cell activation. Thus, by immunizing with cells coated with Bi-MAbs specific for CD28, the interactions between B7 on such cells and CTLA-4 (or other T cell activation down-regulating molecules) is minimized and/or bypassed. In addition, Bi-MAbs reactive with other costimulatory T cell surface molecules (e.g., CD2, CD48) may also be used in the practice of the method of the invention. Furthermore, more powerful 15 costimulation may be achieved by using a multiplicity of Bi-MAbs having specificity for various T cell costimulators.

Bridging substances may be prepared using well known technologies. As shown in the examples which follow, Bi-MAbs may be used effectively as the bridging substance. Such Bi-MAbs may be generated using methods well known in the art, such as, for example, 20 those described in Example 2, and as described in the references cited therein. Bi-MAbs containing multiple T cell costimulatory molecule binding sites may be prepared by chemical linkage in order to provide a means for generating multiple costimulatory activation circuits.

In addition to Bi-MAbs, molecules engineered to contain functional binding sites 25 specific for both the antigen(s) of the target cell(s) and the T cell costimulatory molecule(s) may be used as the bridging substance in the practice of the method of the invention. Such molecules may, for example, comprise proteins, other macromolecules, and polymers engineered to contain the desired binding sites, and may be prepared by using genetic engineering technologies, synthetic technologies, or by chemical linkage of component polypeptides, polymers, and/or other macromolecules. The binding site components of such 30 bridging substances may comprise, for example, Fab2 antibody fragments, antibody binding sites, natural or engineered ligands, or other factors reactive with the target cell antigen(s) and

T cell costimulatory molecule(s).

The bridging substance may be administered to a patient *in vivo* using a pharmaceutically acceptable carrier or a variety of drug delivery systems well known in the art. As an example, for cancer immunotherapy, a combination of the cytokines TNF α and 5 IFN γ and an anti-CD28 Bi-MAb may be formulated within a controlled release preparation which is administered to the patient by directly injecting the preparation into the lymph nodes or into the tumor itself.

The method of the invention is particularly useful in treating cancers. This aspect of the invention is more fully described by way of the examples presented in Example 6. The 10 data presented in the examples indicate that strongly immunogenic tumor cells can be generated using the two-step method of the invention, comprising (1) *in vitro* treatment of autologous tumor cells with a combination of γ -interferon (IFN γ) and tumor necrosis factor- α (TNF α), and (2) pre-incubation with a Bi-MAb specific for both an antigen on tumor cells and CD28 on T cells. The resulting modified tumor cells are able to act as a cellular vaccine 15 that elicits CTL-mediated immunity which can both prevent and cure established tumors. Autologous cancer cells and other diseased cells can be removed from patients by surgery or other techniques, such as fine needle biopsy, including, but not limited to, devices and methods described in U.S. Patents 5,669,394, 5,655,541, 5,241,969, 5,060,658, 4,989,614, 4,697,600, and 4,605,011, incorporated by reference herein.

20 In particular, the studies described in the examples which follow show that cytokine-treated, anti-CD28 Bi-MAb-armed hepatoma cells induce protective immunity against parental tumor cell challenge and, moreover, cure established gross hepatomas in mice. In addition, the studies described in Example 7, show that the method of the invention also induces protective immunity against lymphoma and colon carcinoma.

25 Different routes of administration, or combinations thereof, may be preferred when treating different cancers or other diseases using the cellular vaccines of the invention. As illustrated by the study briefly described in Example 4, immunization with cytokine-treated (and un-armed) cells followed by intravenous administration of an anti-CD28 Bi-MAb induces some anti-tumor immunity in the hepatoma model system.

30 In comparison, as shown by the results of the studies described in Examples 5 and 6, the administration of cells treated with cytokines *in vitro* and armed with Bi-MAbs *in vitro*

induces uniform tumor immunity and cures established hepatomas. It is possible that insufficient localization of the Bi-MAbs to the tumor tissue following intravenous injection in the former case is responsible for the difference in therapeutic efficacy.

Individuals may be immunized against a variety of diseases with cytokine-treated, 5 anti-CD28 Bi-MAb-armed autologous cells, thereby providing the individual's immune system with a signal sufficient to activate T cells and confer protective immunity. Similarly, individuals may be treated for a variety of diseases by administering cytokine-treated, anti-CD28 Bi-MAb-armed autologous cells of the disease or lesion, thereby providing the individual's immune system with a signal sufficient to activate T cells and induce a cytotoxic 10 T lymphocyte response. In both cases, Bi-MAbs with a specificity for other T cell costimulatory molecules may be used to arm the treated cells with a means to physically bridge to T cells *in vivo*.

In addition to MHC class I, ICAM-1, ICAM-2 and VCAM-1 molecules, treatment 15 with cytokines may enhance tumor antigen processing by tumor cells, and may induce the expression of other cell surface molecules essential for T cell activation. A combination of cytokine treated hepa 1-6 cells and immobilized anti-CD28 MAb failed to stimulate splenic T cells *in vitro* or to induce anti-tumor immunity *in vivo* in applicant's model system. This suggests that the signal delivered by the interaction between CD28 and anti-CD28 MAb is not sufficient in itself to induce T cell activation.

20 In contrast, a strongly immunogenic response is obtained when cytokine treated tumor cells armed with anti-CD28 Bi-MAb *in vitro* interact with CD28 on T cells, indicating that a physical bridging function is an important component of the activation process.

In addition, the observation that cytokine treated, B7 transfected hepa 1-6 cells were 25 not able to activate splenic T cells *in vitro* (Example 2) is consistent with the recent finding that B7 may interact with CTLA-4 to deliver a negative regulatory signal, and provides a strong rationale for using anti-CD28 Bi-MAbs to physically link the antigen presenting cell specifically to CD28 molecules for T cell activation.

The invention provides an effective alternative to gene transfer and tumor:APC engineering for the development of cellular vaccines. In particular embodiments of the 30 invention, described in the following examples, the attachment of Bi-MAbs to tumor or other target cells takes place *in vitro*. Accordingly, the antigens on such cells need not be unique to

those cells. Essentially, any antigen may be targeted. Bi-MAbs can be produced by linking anti-CD28 MAbs to Mabs that recognize any antigen expressed on the tumor or other target cell, including antigens which are also expressed on large populations of cells in the individual to be treated (e.g., lymphocytes). This approach may be particularly useful in 5 situations where Mabs to tumor specific antigens are not available.

EXAMPLE 1

CYTOKINE INDUCED EXPRESSION OF ADHESION AND MHC MOLECULES ON HEPA 1-6 CELLS *in vitro*

10 Hepa 1-6 is a chemically induced hepatoma originating in a C57BL/6 mouse (G. J. Darlington et al., 1980, *J. Natl. Cancer Inst.* 64: 809). Cells derived from this tumor grow rapidly and form subcutaneous nodules in syngeneic animals. Hepa 1-6 cells lack both MHC class I and B7 molecules on their cell surfaces and do not induce a host immune response even when transfected with genes encoding the B7-1 or B7-2 molecule.

15 The conditions and cytokines most optimal for the amplification of activation signals on hepa 1-6 cells were determined as follows. One ml of Hepa 1-6 cells was plated into 24 well tissue culture plates at a concentration of 2×10^6 cells / ml and incubated with either IFN γ , 100 U, or TNF α , 50 U, or a combination of IFN γ and TNF α at these same concentrations in complete RPMI-1640 medium supplemented with 10 % fetal calf serum, 20 2mM glutamine, 1x non-essential amino acid and 1 mM sodium pyruvate for 48 hr at 37°C. Hepa 1-6 cells incubated similarly but in medium alone were used as control.

Cells were washed with phosphate-buffered saline (PBS) and stained with rat monoclonal antibodies to mouse MHC class I (M1/42), MHC class II (M5/114), CD44 (KM81) (ATCC), ICAM-1 (HA58), ICAM-2 (3C4) and VCAM-1 (51-10C9) (PharMingen, 25 San Diego, California). To stain for mouse B7-1 (CD80) and B7-2 (CD86), we used CTLA4-Ig, a soluble fusion protein containing the variable domain of the human CTLA4 protein and the hinge, CH2, and CH3 domains of the human IgG1 constant region (Y. J. Guo et al., 1994, *Science* 263: 518; C. Caux et al., 1994, *J. Exp. Med.* 180: 1841; E. Murphy et al., 1994, *J. Exp. Med.* 180: 223; R. Seder et al., 1994, *J. Exp. Med.* 179: 299; B. Blazar et al., 1994, *Blood* 83: 3815; K. Hathcock et al., 1993, *Science* 262: 905; P Linsley et al., 1991, *J. Exp. Med.* 174: 561).

Cells were incubated with the antibodies or chimeric protein for 40 minutes on ice. A rat antibody to mouse CD3 (YCD3) and a soluble human CD44-Ig chimeric protein were used as a negative controls. Cells were washed three times. Fluorescent isothiocyanate (FITC)-conjugated goat antibody to rat Ig or FITC- labeled rabbit antibody to human Ig was 5 added for an additional 40 minutes on ice. Samples were then washed, fixed and analyzed in a FACScan (Becton Dickinson, San Jose, California). The mean fluorescent intensity in the negative control group (medium alone) was at background level except for ICAM-1.

Hepa 1-6 cells incubated with a combination of interferon (IFN γ) and tumor necrosis factor (TNF α) showed expression of MHC class I, intercellular adhesion molecule 2 (ICAM-10 2) and vascular adhesion molecule 1 (VCAM-1), and showed significantly enhanced expression of intercellular adhesion molecule 1 (ICAM-1) (FIG. 1). This pattern of expression was maintained for more than three days *in vitro* after removing cytokines from the medium. However, the cytokine treated hepa 1-6 cells (CT-hepa 1- 6) were still able to 15 form tumors in syngeneic animals. It was assumed that this may be because the cells were deficient in providing a CD28 mediated costimulatory signal due to absence in expression of B7.

EXAMPLE 2

STIMULATION AND PROLIFERATION OF SYNGENIC SPLENIC T-CELLS INDUCED BY CYTOKINE TREATED HEPA 1-6 CELLS AND ANTI-CD28 Bi-MAb *in vitro*

The following example demonstrates that cytokine activated Hepa 1-6 cells in combination with Bi-MAbs to CD28 and tumor cell antigens stimulate proliferation of splenic T cells *in vitro*, indicating that such Bi-MAbs can provide a CD28 costimulatory 25 signal. Four Bi-MAbs, CD28:gp55, CD28:gp95, CD28:gp115 and CD28:gp210, each with one binding specificity for the CD28 molecule on T cells and a second binding specificity for one of three glycoproteins expressed on tumor cell surfaces, were prepared and used as follows.

For preparation of Mabs and Bi-MAbs, Wistar rats were immunized with 2×10^7 30 Hepa 1-6 cells in CFA. Following three additional boosts with the same cells in ICFA over an 8 week period, spleen cells from immunized rats were fused with YB2/0 rat myelomas as

previously described (J. Alan & T. Robin, in: *Immunochemistry in Practice*, Chapter 2 (Blackwell, New York, 2d ed. 1988)). More than twenty Ig-producing hybridomas were selected by immunofluorescent staining. Three antibodies reacted with hepa 1-6 cells by flow cytometry analysis. These Mabs separately recognized a 55 Kd, 95 Kd, 115 Kd or 210 Kd glycoprotein expressed on most tumor cells as determined by immunoprecipitation. The 5 Mabs were designated as anti-gp55, anti-gp95, anti-gp115 and anti-gp210, respectively.

Anti-mouse CD28 Mabs were generated by first immunizing Wistar rats with a mouse T cell hybridoma cell line expressing high levels of CD28 antigen on the cell surface. After cell fusion, hybridomas producing anti-CD28 MAb were selected with immunofluorescent 10 analysis by FACScan. Anti-CD28 Mabs were further characterized and confirmed by immunoprecipitation and T cell proliferation and IL-2 production assays.

Hybridoma producing anti-mouse CD18 used to generate CD18:gp55 Bi-MAb was purchased from ATCC. All Mabs used in these experiments were purified by passage of ascites from nude mice over a protein G column.

15 Bi-MAbs were produced from these Mabs as previously described (J. A. MacLean et al., 1993, *J. Immunol.* 150: 1619; L. K. Gilliland et al., 1988, *Proc. Natl. Acad. Sci. USA* 85: 7719; C. Bode et al., 1989, *J. Biol. Chem.* 264: 944).

Normal splenic T cells were purified by nylon wool column. Purified T cells (5×10^6 /well) were co-cultured in complete RPMI-1640 medium at 37°C for 96 hours with 5×10^5 20 irradiated (5000 roentgens) cytokine treated (as described in Example 1) or untreated hepa 1-6 cells in the presence or absence of CD28:gp55, CD28:gp95 or CD28:gp210 Bi-MAb. A CD18:gp55 Bi-MAb that bridges CD18 on T cells to gp55 on tumor cells and a mixture of parental CD28 plus gp55 Mabs were used as controls. Cytokine treated or untreated hepa 1-6 cells transfected with B7 gene and expressing high level of B7 molecules on cell surfaces 25 were also used as a control. The percentage of $CD3^+CD8^+CD25^+$ T cells (mean \pm SD) was determined by three color analysis in FACScan using Cy-ChromTM labeled anti-CD3, PE-labeled anti-CD8 and FITC-conjugated anti-CD25 antibodies (PharMingen, San Diego, California).

Each of the Bi-MAbs was tested both *in vitro* and *in vivo* for its ability in combination 30 with cytokine treatment of hepa 1-6 cells to activate tumor specific CTLs. Mouse splenic cells were cocultured with either cytokine treated or untreated hepa 1-6 cells in the presence

of purified anti-CD28 Bi-MAb or control antibody, 50 mg/ml each, at 37°C for 9 days.

The results presented in FIG. 2 indicate that the combination of cytokine treated tumor cells and any one of the three anti-CD28 Bi-MAbs significantly stimulated splenic T cell proliferation.

5 No stimulation was obtained in the absence of either anti- CD28 Bi-MAbs or the cytokine treated autologous tumor cells. Interestingly, hepa 1-6 cells transfected with, and expressing high levels of B7, were not effective in stimulating naive T cells *in vitro* when treated with the cytokines in similar manner. The majority of lymphocytes generated by this approach were CD3⁺CD8⁺CD25⁺ T cells.

10

EXAMPLE 3

**In vitro CYTOTOXICITY OF CTLs GENERATED BY
CYTOKINE TREATED HEPA 1-6 TUMOR CELLS
IN COMBINATION WITH ANTI-CD28 Bi-MAbs**

15 Cytotoxicity of CTLs generated by *in vitro* priming of naive splenic T cells with cytokine treated hepa 1-6 cells in combination with anti-CD28 Bi-MAbs or control MAb was established as follows. Nylon wool-enriched naive splenic T cells (5×10^6 /well) were first stimulated *in vitro* by incubation with 5×10^5 irradiated (5000 roentgens) cytokine treated hepa 1-6 cells (as described in Example 1) in combination with anti- CD28 Bi-MAbs, control 20 antibodies or irradiated B7+ hepa 1-6 alone at 37°C for 9 days. γ -irradiated naive splenic cells (5×10^6) were added into cultures as feeder cells. At the 3rd and 6th day after stimulation, 2 ml of complete RPMI-1640 medium containing recombinant human IL-2 (20 U) were added into each culture well separately.

25 Cytotoxicity of CTLs toward syngeneic, allogenic tumor cells and a NK sensitive YAC-1 cell line was determined in a standard 4 hour ^{51}Cr release assay. Results from three experiments are shown in FIG. 3 as the percentage of ^{51}Cr release at 1:20 E:T ratio as measure of tumor cell lysis (mean \pm SD). These results show that CTLs generated as described herein have cytolytic activity specific to autologous tumor cells.

EXAMPLE 4**EFFECT OF INTRAVENOUSLY ADMINISTERED ANTI-CD28 Bi-MAbs**
ON TUMORIGENICITY OF CYTOKINE TREATED HEPA 1-6 CELLS

Mice injected with cytokine treated hepa 1-6 cells followed by intravenous

5 administration of anti-CD28:gp55 Bi-MAb at a dose of 100 ug on day 1, 2 and 4, experienced
delayed tumor formation and 40 percent of these mice (8/20) had tumor regression. Animals
injected with a combination of parental hepa 1-6 cells and anti-CD28 Bi-MAb or a
combination of CT-hepa 1-6 cells and control antibody all developed tumors and died within
60 days after the inoculation of tumor cells. Immunohistological studies at 24, 48, and 72 hr
10 after injection of the Bi-MAbs showed that the Bi-MAbs were widely distributed in lungs,
liver and kidney in addition to tumor tissue.

EXAMPLE 5**INDUCTION OF PROTECTIVE IMMUNITY *in vivo* WITH CYTOKINE**
TREATED HEPA 1-6 TUMOR CELLS ARMED WITH ANTI-CD28 Bi-MAbs

15 Tumor cells were first treated *in vitro* with a combination of IFN γ , 100 U, and TNF α ,
50 U, in RPMI-1640 medium with 10% fetal calf serum at 37°C, 5% CO₂ for 48 hours. Cells
were then washed with Phosphate-Buffered Saline, PH 7.4 (PBS) x 3 at 20°C and incubated
with anti-CD28 Bi-MAbs at a concentration of 50 ug/ml on ice for 45 min as described in

20 Example 1. After an additional incubation in an equal volume of 30% polyethylene glycol
(PEG) in RPMI-1640 for 60 minutes at 4°C, the cells were washed x3 as described above and
suspended in a final concentration of 1-2 x 10⁷ / ml PBS. To arm cells with Bi-MAbs or
Mabs, cytokine treated or untreated parental tumor cells were pre-incubated with respective
antibodies for 45 minutes.

25 Five groups of C57BL/6 mice, 5 per group, were immunized subcutaneously with 1 x
10⁶ cytokine treated hepa 1-6 cells armed with CD28:gp55, CD28:gp95, CD28:gp 210 or a
control Bi-MAb CD18:gp55, or with 1 x 10⁶ untreated hepa 1-6 cells armed with CD28:gp55
Bi-MAb. After two weeks, mice in each of the groups were challenged with 2.5 x 10⁶
parental hepa 1-6 cells injected subcutaneously.

30 Cytokine treated hepa 1-6 tumor cells (CT-hepa 1-6) pre- incubated/armed with anti-
CD28 Bi-MAbs completely lost their ability to form tumors in syngeneic mice, whereas

cytokine treated hepa 1-6 cells pre-incubated/armed with control antibodies retained their tumor forming capacity. The results of the immunization experiment are shown in FIG. 4. Mice immunized with CT-hepa 1-6 cells armed with each of the CD28/tumor antigen Bi-MAbs developed protective immunity against challenge with parental tumor cells, and all of 5 these animals remained tumor- free for 120 days after such challenge (FIG. 4). In contrast, all mice injected subcutaneously with either untreated Hepa 1-6 cells armed with anti-CD28 Bi-MAbs or CT-hepa 1-6 cells armed with the control antibody CD18:gp55 developed tumors and died within 50 days following challenge with the parental hepa 1-6 cells (FIG.4). This experiment was repeated twice with comparable results.

10

EXAMPLE 6

APPLICATION OF THE METHOD OF THE INVENTION TO THE HEPATOMA CANCER SYSTEM: CURE OF ESTABLISHED HEPATOMAS WITH CYTOKINE TREATED, Bi-MAb-ARMED HEPA 1-6 CELLS

15 Vaccination

To establish that immunization with CT-hepa 1-6 cells armed with anti-CD28 Bi-MAbs can cure established hepatomas, the following three studies were performed, each of which indicates that the therapeutic administration of Bi-MAb-armed, cytokine treated hepa 1-6 cells is an effective therapy for hepatoma.

20 In the first study, forty mice were inoculated subcutaneously with 2×10^6 wild type hepa 1-6 cells. Fourteen days later, after the development of microscopic tumors, the mice were divided into four groups of ten each. The groups were treated subcutaneously with 2×10^6 cytokine treated or untreated hepa 1-6 cell armed with either the CD28:gp55 Bi-MAb or a control CD18:gp55 Bi-MAb.

25 The results of this study are shown in FIG. 5. All mice in the group treated with CT- Hepa 1-6 cells armed with anti-CD28 Bi- MAb survived for more than 100 days (FIG 5). In contrast, all mice in the group treated with control Bi-MAb-armed/cytokine treated hepa 1-6 cells, and all mice in the groups treated with untreated hepa 1-6 cells armed with the CD28:gp55 Bi-MAb or the CD18:gp55 control Bi-MAb, died within about 40 days (FIG. 5).

30 This experiment was repeated twice with comparable results.

In the second study, five groups of five mice each were injected subcutaneously with

1 $\times 10^6$ hepa 1-6 cells. After four weeks, mice bearing tumors 6-8 mm (in the greatest dimension) were injected subcutaneously with 1 $\times 10^6$ cytokine treated hepa 1-6 cells armed with the CD28:gp55, CD28:gp95 or CD28:gp210 Bi- MAbs, respectively¹, and then injected subcutaneously with a boost of the Bi-MAb-armed/cytokine treated hepa 1-6 cells at the same 5 dose 7 days thereafter. Cells pre-incubated with CD18:gp55 or mixed with both anti-CD28 and anti-gp55 parental Mabs (CD28+gp55) at a concentration of 50 ug each were used as controls. Tumor size was periodically measured.

The results of this study are shown in FIG. 6. Hepatomas in the mice treated with the CD28:gp55, CD28:gp95 and CD28:gp210 Bi- Mabs regressed to undetectable size within 10 about 40 days (FIG. 6). In contrast, hepatomas in the mice treated with the CT-hepa 1-6 cells pre-incubated with either control Bi-MAb or MAb more than doubled in size within the same period, and all of these mice within that period (FIG. 6). This experiment was repeated three times with comparable results.

In the third study, γ -irradiated Bi-MAb armed tumor cells were used as the vaccine. 15 Three groups of five mice were inoculated subcutaneously with 1 $\times 10^6$ hepa 1-6 cells. After two weeks, mice were then injected subcutaneously either with 1 $\times 10^6$ γ -irradiated, cytokine treated hepa 1-6 cells armed with CD28:gp55 Bi-MAb, or with a combination of γ -irradiated, cytokine treated hepa 1-6 cells plus a mixture of parental anti-CD28 and anti-gp55 Mabs, or with 1 $\times 10^6$ γ -irradiated hepa 1-6 alone.

20 The results of this study, shown in FIG. 7, are similar to the results obtained in the second study described above. Only the mice treated with the γ -irradiated, cytokine treated hepa 1-6 cells armed with CD28:gp55 Bi-MAb survived (for more than 100 days). All mice in the other two groups died within 40 days of treatment.

Upon examination, tumor tissue from mice first injected with the parental tumor cells 25 and then given cytokine treated autologous tumor cells pre-incubated with anti-CD28 Bi-MAb showed marked inflammatory responses with abundant lymphocyte infiltration. In accordance with *in vitro* stimulation data, the majority of the infiltrating lymphocytes were

¹ hepa 1-6 can also be coated with two or more different anti-CD28 Bi-MAbs, e.g., both anti-CD28xgp55 and anti-CD28xgp95. Because hepa 1-6 cells have similar levels of expression of gp55, gp95 and gp210 antigens, the number of anti-CD28 Bi-MAbs coating the cells preincubated with two or more different anti-CD28 Bi-MAbs should be significantly higher than the pre-incubated with one anti-CD28 Bi-MAb.

CD3⁺CD8⁺ CD25⁺ T cells, as determined by immunofluorescent staining of tissue sections with rat anti-mouse CD3, CD8 and CD25 MAbs. There was no local immune response in mice injected either with untreated tumor cells armed with anti-CD28 Bi-MAb or with cytokine treated tumor cells armed with control Bi-MAbs.

5 To further investigate if the induced immunity was mediated by CTLs, mice were depleted of CD8⁺ T cells by antibody treatment before or after immunization. Depletion of CD8⁺ T cells either before or after immunization abrogated the ability of the cellular vaccine to elicit anti-tumor immunity *in vivo*.

10 20-25% of infiltrating cells was CD3⁺CD25⁺CD4⁺ T cells. Hepa 1-6 cells were able to form tumors in 40 % of mice that were depleted CD4⁺ T cells. Therefore, induction of protective tumor specific immunity in this system requires both CD4⁺ and CD8⁺ T cells. After induction of protective immunity, CD8⁺ cells can mediate tumor cell destruction alone.

Adoptive Transfer of CD8⁺ T cells from immunized mice

15 To directly demonstrate that CD8⁺ T cells from immunized mice can eradicate hepa 1-6 tumors, an adoptive transfer experiment was carried out in nude mice to reduce the numbers of CD8⁺ T cells required for adoptive transfer. Three groups of mice were injected subcutaneously with 1×10^6 hepa 1-6 tumor cells. Two weeks after tumor cell injection, one group of mice received 1×10^7 CD8⁺ T cells obtained from normal C57BL/6 mice. The second group received identical numbers of CD8⁺ T cells from mice immunized with cytokine treated, anti-CD28 Bi-MAb armed hepa 1-6 cells 10 days earlier. The third group received CD8⁺ T cells from mice immunized with cytokine treated hepa 1-6 cells pre-incubated with CD18:gp55 Bi-MAb. All mice injected with hepa 1-6 and received normal CD8⁺ T cells or received CD8⁺ T cells from CD18:gp55 armed, cytokine treated hepa 1-6 cells died within 50 days after tumor injection. In contrast, all mice injected with parental hepa 1-6 cells and received CD8⁺ T cells from mice immunized with cytokine treated, anti-CD28-Bi-MAb armed hepa 1-6 cells remained tumor free for more than 100 days.

25 30 In this experiment, the spleens from normal mice, mice immunized with cytokine treated hepa 1-6 cell armed with CD18:gp55 Bi-MAbs or mice immunized with cytokine treated hepa 1-6 cells armed with CD28:gp55 Bi-MAb were removed and single cell suspensions prepared. CD8⁺ T cells were enriched by panning of the spleen cells with plastic

dishes coated with Mab specific for mouse CD8. More than 80% of the enriched lymphocytes are CD8⁺ T cells as judged by immunofluorescent staining and analysis with FACScan.

5

EXAMPLE 7

APPLICATION TO ADDITIONAL CANCER SYSTEMS: INDUCTION OF PROTECTIVE IMMUNITY AGAINST LYMPHOMA AND COLON CARCINOMA USING CYTOKINE TREATED, Bi-MAb-ARMED CANCER CELLS

The immunogenicity of cytokine treated, autologous tumor cells pre-incubated with 10 anti-CD28 Bi-MAbs was tested in two additional cancer systems, EL-4 lymphoma and SMCC-1 colon carcinoma.

The EL-4 lymphoma becomes immunogenic when transfected with the B7 gene. In contrast, SMCC-1 colon carcinoma remains non- immunogenic even after transfection with the B7 gene. Both of these cell lines grow rapidly and develop subcutaneous tumors in 15 syngenic C57 BL/6 mice (see, for example, Li et al., 1996, J. Exp. Med. 180: 211). Both of these cell lines express the gp55 antigen on their cell surfaces. Accordingly, the anti-CD28:gp55 Bi-MAb described in the previous examples was also used in these studies.

Three groups of mice were immunized subcutaneously with 1×10^6 cytokine treated, CD28:gp55 Bi-MAb-armed, hepa 1-6, EL-4 or SMCC-1 tumor cells respectively.

20 After two weeks, mice immunized with the modified hepa 1-6 cells were divided into three groups and challenged by a subcutaneous injection with 1×10^6 hepa 1-6 cells, SMCC-1 cells or EL-4 cells respectively.

The mice immunized with the cytokine treated, CD28:gp55 Bi- MAb-armed SMCC-1 cells were divided into two groups. One group was challenged by subcutaneous inoculation 25 with 1×10^6 SMCC-1 cells. The other group was challenged by subcutaneous inoculations of EL-4 cells and 1×10^6 hepa 1-6 cells into the left and right flanks of mice, respectively.

Similarly, the mice immunized with the cytokine treated, CD28:gp55 Bi-MAb-armed EL-4 cells were divided into two groups which were challenged with either EL-4 cells alone or with both SMCC-1 and hepa 1-6 cells.

30 The results of this study, presented in Table I below, were repeated twice with identical results.

TABLE I
Specificity of the immune responses elicited by
cytokine treated and anti-CD28 Bi-MAb armed tumor cells.

IMMUNIZATION CELLS	CHALLENGE CELLS	NUMBER OF MICE WITH TUMORS
Bi-MAb-CT Hepa 1-6	Hepa 1-6	0 of 10
Bi-MAb-CT Hepa 1-6	SMCC-1	6 of 6
Bi-MAb-CT Hepa 1-6	EL-4	5 of 5
Bi-MAb-CT SMCC-1	SMCC-1	0 of 6
Bi-MAb-CT SMCC-1	EL-4 + Hepa 1-6	6 of 6
Bi-MAb-CT EL-4	EL-4	0 of 6
Bi-MAb-CT EL-4	SMCC-1 + Hepa 1-6	6 of 6

5 Immunization with cytokine treated EL-4 (CT-EL-4) or cytokine treated SMCC-1 (CT-SMCC-1) tumor cells armed with the anti-CD28:gp55 Bi-MAb elicited anti-tumor immunity against autologous parental tumor in all animals (Table 1).

10 The elicited immunity in all three experimental groups was tumor-specific. For example, immunization with CT-hepa 1-6 cells armed with anti-CD28 Bi-MAb did not inhibit growth of syngeneic EL-4 or SMCC-1 tumors *in vivo* (Table 1). Interestingly, in the absence of treatment with cytokines, the parental EL-4 cells expressed high levels of MHC class I at cell surfaces, yet when pre-incubated with anti-CD28:gp55 Bi-MAb, they were still not able to induce protective immunity. In addition, CTLs from mice immunized with cytokine treated autologous tumor cells armed with anti-CD28 Bi-MAbs specifically lysed the parental tumor cells but not other tumor cells *in vitro*.

EXAMPLE 8

INDUCTION OF CELLULAR IMMUNITY BY TUMORS ARMED WITH
MULTIVALENT BRIDGE MOLECULES

20 The above data showed that SMCC-1, EL-4 and Hepa1-6 cells became more immunogenic after treatment *in vitro* with a combination of cytokines and anti-CD28 bispecific monoclonal antibody. The modified tumor cells were able to elicit anti-tumor specific immunity that were both preventive and curative.

The following data showed that EL-4 and SMCC-1 cells were also effective for eliciting preventive and curative antitumor immunity in syngenic animals without cytokine treatment when these cells were precoated with two different bispecific monoclonal antibodies (Bi-MAbs), one specific for CD28 and another specific for 4-1BB.

5 4-1BB is a glycoprotein expressed on primed CD4⁺ and CD8⁺ T cells. 4-1BB signaling either by binding to 4-1BB ligand or by antibody ligation delivers a dual mitogenic signal for T-cell activation and growth (Alderson et al., 1994, *Eur. J. Immunol.* 24:2219; Hurtado et al., 1995, *J. Immunol.* 155:3360; and DeBenedette et al., 1995, *J. Exp. Med.* 181:985). It was shown that administration of anti-4-1BB monoclonal antibodies could 10 eradicate established large tumors in mice (Melero et al., 1997, *Nat. Med.* 3:682).

The cells were coated *in vitro* with anti-gp55:anti-CD28 and anti-gp115:anti-4-1BB Bi-MAbs in a concentration of 50 ug/ml on ice for 45 min. After fixed with PEG and washed for three times, the tumor cells coated with the two different Bi-MAbs were subcutaneously injected into syngenic animals at different doses. Two weeks later, the immunized animals 15 were challenged with parental tumor cells and tumor formation rate was observed (Table II).

Table II. Comparison of the efficacy of immunogenic tumor cells modified with different process *in vitro* on eliciting preventive antitumor immunity *in vivo*

	<u>Immunization</u>	<u>Dose</u>	<u>Challenge</u>	<u>Tumor Formation</u>
20	Irradiated EL-4 Wt	1x10 ⁶	1x10 ⁶ EL-4 Wt	100%
	Irradiated CT-EL-4 Wt	1x10 ⁶	1x10 ⁶ EL-4 Wt	100%
	Irradiated EL-4 coated w/ CD28BiMab	1x10 ⁶	1x10 ⁶ EL-4 Wt	50%
25	Irradiated CT-EL-4 coated w/ CD28BiMab	1x10 ⁶	1x10 ⁶ EL-4 Wt	0%
		1x10 ⁵	1x10 ⁶ EL-4 Wt	20%
		5x10 ⁴	1x10 ⁶ EL-4 Wt	60%
30	Irradiated EL-4 Wt coated w/ anti-CD28&anti-4-1BB BiMabs	1x10 ⁶	1x10 ⁶ EL-4 Wt	0%
		1x10 ⁵	1x10 ⁶ EL-4 Wt	0%
		5x10 ⁴	1x10 ⁶ EL-4 Wt	10%

In curative experiments, syngenic animals were first inoculated subcutaneously with 2x10⁶ parental tumor cells. After two to four weeks, the tumor bearing animals were injected

with the modified tumor cells. The mean survival time was monitored (Table III).

Table III. Comparison of the efficacy of immunogenic tumor vaccines armed with monovalent or multivalent bi-specific Mabs on eliciting curative antitumor immunity *in vivo*

5

Tumors	Vaccination	Dose	Animal Survival (%)*)
SMCC-1	SMCC-1 Wt	1x10 ⁶	0%
10	SMCC-1	1x10 ⁶	100%
		5x10 ⁵	30%
SMCC-1	SMCC-1+CD28 & 4-1BB BiMabs	1x10 ⁶	100%
		5x10 ⁵	80%
15	SMCC-1	SMCC-1+CD28& 4-1BB BiMabs (multivalent BiMabs)	
		1x10 ⁶	100%
		5x10 ⁵	100%
		1x10 ⁵	60%

* 60 day survival rate after tumor vaccine treatment.

20

EXAMPLE 9

GENERATION OF CELLULAR IMMUNITY AGAINST VIRUS INFECTED CELLS

Primary liver cells were obtained from clinical biopsy laboratory and cultured in hepatocellular media. Autologous peripheral blood lymphocytes were obtained from same patients during operation and cultured in complete RPMI-1640 medium supplemented with 5% human AB serum and 5% fetal calf serum, 20 iu/ml rh-IL-2.

Liver cells were infected by E1B deleted Adenovirus as reported previously. Virus infection was confirmed by RT-PCR and histological examination.

Infected liver cells were then treated *in vitro* with (1) cytokines alone, (2) BiMabs alone, or (3) cytokine + bispecific Mabs. The modified virus-infected liver cells were irradiated at a dose of 5000R and were then co-cultured with autologous PBL in complete RPMI-1640 medium as reported previously.

The cytotoxicity of the CTLs generated by the unmodified and the modified liver cells was determined with a standard 4 h ⁵¹Cr release assay.

Table IV. Generation of cytotoxic T cells *in vitro* by stimulating autologous peripheral blood lymphocytes with virus-infected human fetal liver cells pretreated with a combination of INF γ and TNF α and coated with anti-CD28 bispecific monoclonal antibody

	<u>Target cells</u>	<u>Treatment</u>	<u>Effectors</u>	<u>E:T Ratio</u>	<u>Cytotoxicity</u>
5	Liver cells	None	PBL	1:50	~3%
	Liver cells	Cytokines	PBL	1:50	~3%
	Liver cells	BiMabs	PBL	1:50	~3%
10	Liver cells	Cyto+BiMab	PBL	1:50	~3%
	Ad-Liver cells	None	PBL	1:50	~3%
	Ad-Liver cells	Cytokines	PBL	1:50	~5%
	Ad-Liver cells	BiMabs	PBL	1:50	~13%
	Ad-Liver cells	Cyto+biMabs	PBL	1:50	~20%
15	Ad-Liver cells	biMabs (Multivalent*)	PBL	1:50	~40%
	Ad-Liver cells	Cyto+biMabs(Multivalent*)	PBL	1:50	~40%

20 •Anti-Gp115:Anti-CD28 bispecific monoclonal antibody was generated by chemical linking several anti-CD28 and anti-Gp115 monoclonal antibodies together and purified by sequential affinity columns, one specific binding gp-115 and another for CD28 monoclonal antibody specifically.

25

EXAMPLE 10

GENERATION OF HUMANIZED Bi-MAbs

The Bi-MAbs of this invention can be generated by a variety of techniques known to those skilled in the art (see, Vaughan et al., Nature Biotechnology 16:1015 – 1016; 30 Hoogenboom, 1998, Immunotechnology 4:1-20; and Holliger & Hoogenboom, 1998, Nature Biotechnology 16:1015). (1) Recombinant chimeric antibodies comprise rodent variable (V) regions attached to human constant (C) regions. (2) In humanized (or reshaped) antibodies, only the antigen-binding complementarity-determining region (CDR) loops derive from rodent antibodies. (3) All-human antibodies can be constructed from human V regions 35 (isolated by phage display technology) and human C regions. Fv and scFv fragments, and diabodies comprise only V regions and can be cloned from hybridomas or isolated from

phage libraries. (4) All-human antibodies can also be prepared from transgenic non-human animals capable of producing human antibodies. Therefore, the techniques described in the following references can be readily used to prepare Bi-MAbs for purposes of this invention: Merchant, 1998, Nature Biotechnology 16:677; Holliger, 1997, Nature Biotechnology 15:632; McGuinness et al., 1996, Nature Biotechnology 14:1149; Reiter et al., Nature Biotechnology 14:1239; Vaughan et al., 1996, Nature Biotechnology 14:309; WO 98/24884; U.S. Patents 5,814,318, 5,789,215, 5,770,429, 5,693,762, 5,530,101, 5,637,481, 5,601,819, and 5,141,736.

For example, anti-CD28 and anti-4-1BB monoclonal antibodies that costimulate human T cells can be humanized for T cell activation and the induction of antitumor immunity both *in vitro* and *in vivo*. Variable (V) domains of mouse Mab can be spliced to join the constant (C) domains of human IgG by PCR technology. The transfected cell lines producing high level of humanized Mab will be selected and the Mab will be purified to milligram quantities for testing efficacy in T cell activation *in vitro* and antitumor effect *in vivo*. Specifically, two different bispecific monoclonal antibodies will be generated and tested, antitumor antigen X CD28 and Antitumor antigen X 4-1BB.

To generate antitumor antigen X CD28 Mab, the cDNA fragment of V region of mouse Ig light (L) and heavy (H) chain is amplified by PCR from the candidate hybridomas producing anti-CD28 or antitumor antigen Mabs and inserted into plasmids that contain human C κ and C γ 1 region driven by CMV and β -globin major promoters, respectively. The vectors also contain neomycin and DHFR resistant gene. The resulting vector will be transfected into DHFR-negative DG44 cells and initially selected by G418 at 400ug/ml. The clones that produce the highest amount of Ig will be further expanded and incubated with methotrexate for gene amplification. The clones resistant to methotrexate will be selected and ELISA will quantitate the Ig production in supernatant. The humanized Mabs will be purified by protein G affinity chromatography. The purified humanized antitumor antigen and antiCD28 Mabs will be used to generate bispecific monoclonal antibodies by either chemical linkage or reconstruction technology under a low salt and low PH condition as described previously.

Alternatively, single chain bispecific monoclonal antibodies (tumor antigen X CD28 and tumor antigen X 4-1BB) can be produced. Two single chain Fv fragments will be joined

through a flexible Gly-Ser linker. The single chain Fv fragment directed against tumor antigen (gp115) is derived from gp115 hybridoma; the other directed against CD28 or 4-1BB is derived from GW28 (CD28) and GW4-1BB (anti4-1BB) hybridomas. The constructs will be expressed in CHO cells as full functional molecules and will be purified via its C-terminal histidine tail on a Ni-NTA (Nitrilotriacetic acid) affinity column.

5 If the cytokine treated human cancer cells (e.g., melanoma and hepatoma cells) armed with humanized antiCD28 or anti-41Bb Bi-MAb can effectively stimulate T cells *in vitro* with generation of CTLs, they can be further tested for anti-tumor immunity *in vivo*. Because of different tumorigenicity of human cancer cells *in vivo*, three xenografted human melanoma 10 and two hepatoma cancer models with similar tumor formation rates in have been established in scid mice and nude mice in our lab and can be used for prevention and cure experiments.

10 For active immunotherapy, scid and nude mice are injected with 5×10^7 autologous peripheral T lymphocytes for reconstitution of a human immune system, which will be confirmed by flow cytometry, T cell proliferation and CTLs assay. The mice will be 15 immunized with 1×10^6 cytokine treated cancer cells armed with humanized antiCD28 or anti4-1BB Bi-MAb for three times with a 10 day period and then challenged with 1×10^6 prenatal tumor cells. For cure experiments, the mice with reconstituted human immune system will be first inoculated with 2×10^6 parental tumor cells. Two weeks after tumor inoculation, 1×10^6 cytokine treated tumor cells armed with Bi-MAb or tumor cells armed 20 with control Bi-MAbs will be injected intravenously into each group of mice bearing tumors. Tumor growth and survival will be monitored.

25 For adoptive immunotherapy, human CD8⁺ clones specific for autologous tumor cells will be generated by stimulating PBL or TILs with cytokine treated cancer cells armed with Bi-MAb *in vitro*. 1×10^7 CTLs will be adoptively transferred into naive or tumor bearing scid mice to evaluate the effectiveness of the T cell clones in prevention and cure of 30 xenografted autologous human melanoma or hepatoma tumors.

If anti-tumor specific immunity can be adoptively transferred by *in vitro* generated CTLs, this will provide an additional opportunity to form a combined active and adoptive anti-cancer immunotherapy for human cancers. Because it has been known that 35 immunosuppression exists in cancer patients especially in the patients with advanced cancers, it is difficult to activate T cells *in vivo* with active vaccine immunotherapy alone for induction

of effective immunity. The combination of active and adoptive therapy can be more effective for elicitation of anti-tumor immunity.

EXAMPLE 11

5 **ARMING FUSION TUMOR VACCINE WITH Bi-MAb TO ENHANCE
IMMUNOGENICITY**

Cellular tumor vaccines can be generated by fusion of tumor cells with antigen processing cells such as activated B cells or dendritic cells. The immunogenicity of fusion tumor vaccine cells can be further enhanced by arming them with antitumor:anti-CD28
10 bispecific monoclonal antibody (e.g., GP55x28, GP95x28, GP115x28 or GP210x28). Without being bound by any theory, applicant proposes that the arming reduces the inhibition of T cell activation by negative signaling from the binding of B7 on fusion cells to CTLA-4 on T cells.

Briefly, fusion tumor vaccines were first produced by fusing rat NBT II tumor cells
15 and mouse SMCC-1 cells with *in vitro* activated syngeneic B cells or *in vitro* activated syngeneic bone-marrow derived dendritic cells using a standard fusion protocol described previously (See, e.g., Guo et al., 1994, Science 263:518-520; WO95/16775). Bispecific monoclonal antibody used in the experiment is GP115xCD28 that was generated by a
chemical approach. After fusion, hybrid tumor cells were selected and armed *in vitro* with
20 bispecific monoclonal antibody. Antitumor antigenxCD18 monoclonal antibody was used as antibody control and fusion tumor vaccine and two-step tumor vaccines were used as cellular vaccine controls. A sub-optimal E:T cell ration was used in the following experiments.

Resulting hybrid tumor cells armed with bispecific monoclonal antibody were co-cultured with syngeneic naïve T cells at different vaccine cell:T cell ratios. The proliferation
25 of T cells was measured by a standard [³H]-Thymidine incorporation assay. All cultures were done in triplicate and the numbers present in the following tables are the average of the radioactive thymidine incorporation in each triplicate.

As showed in Table V, fusion tumor vaccine cells armed with GP115XCD28 Bi-MAb *in vitro* had enhanced ability to stimulate naïve splenic T cells at a 1:50 vaccine:T cell ratio.

In contrast, both fusion tumor vaccine and two-step tumor vaccine achieved similar effect in inducing T cell proliferation at 1:25 ratio.

5 **Table V. Ability of fusion vaccine in stimulating T cell proliferation is significantly enhanced by arming with GP11xCD28 Bi-MAb**

		<u>[³H]-Thymidine incorporation / Cell ratio</u>			
		1:100	1:50	1:25	CD3CD8CD25
Vaccines					
Rat <u>NBT</u> -II B		1,700+400	2,400+380	3,800+1,200	45%
10 Rat <u>NBT</u> -II D		1,980+700	2,250+670	4,100+1,400	41%
<u>Rat</u> NBT-II-B-BiMab		2,400+540	4,900+1400	6,840+1,090	84%
<u>Rat</u> NBT-II-D-BiMab		2,100+680	3,800+980	5,700+1,120	79%
15 <u>Mouse</u> SMCC-1 B		2,240+840	2,800+510	2,890+780	38%
<u>Mouse</u> SMCC-1 D		1,800+470	2,400+650	2,900+920	40%
15 <u>Mouse</u> SMCC-1 B-BiMab		2,700+680	3,300+790	4,900+700	75%
<u>Mouse</u> SMCC-1 D-BiMab		2,300+510	3,100+270	4,400+630	64%

Table V. Normal naïve spleen cells were obtained from syngeneic animals and purified by Nylon wool column. Purified T cells (5×10^6 /well) were co-cultured with 5×10^4 , 1×10^5 or 20 2×10^5 irradiated (5000 roentgens) fused tumor vaccine cells or fused tumor vaccine cells armed with GP115XCD28 BiMab in complete RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 20um/ml recombinant IL-2 for 9 days. The majority of T cells generated by this *in vitro* approach is CD3⁺CD8⁺CD25⁺ T cells indicated by immunofluorescent staining and flow cytometry analysis.

25 Briefly, to stimulate tumor specific CTL responses, purified spleen T cells from naive C57BL/mice were primed with γ -irradiated cytokine treated or untreated hepa 1-6 cells in the presence of Bi-MAbs or control antibodies for 9 days in complete RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 20u/ml recombinant IL-2.

30 Five $\times 10^6$ γ -irradiated syngeneic naive spleen cells were added into cultures as feeders. The cytotoxic activity of *in vitro*-stimulated splenocytes was determined using the ^{51}Cr -release assay. Briefly, tumor or control target cells were labeled with 100 μCi of ^{51}Cr for

60 min and washed three times with HBSS. Labeled target cells were incubated with *in vitro*-primed CTLs at various effector:target ratios in 200 μ l of RPMI-1640 medium containing 10% fetal bovine serum, 2mM glutamine, 0.5% gentamicin sulfate and 2×10^{-5} M 2-mercaptoethanol. After incubating for 4 h at 37°C in 5% CO₂, 200 μ l of culture supernatant 5 was collected and the amount of ⁵¹Cr released from labeled targets was determined. The percentage of cytotoxic activity at each effector:target ratio was calculated as described elsewhere.

10 CTLs generated by these approaches demonstrated specific cytolytic activity to autologous tumor cells as assessed by the ⁵¹Cr releasing assay (Table VI). The results show that CTLs generated by this *in vitro* Bi-MAb modification are tumor specific and have no cross-reaction with syngeneic tumor cells and YAC-1, a NK sensitive cell line.

15 **Table VI. Specific cytotoxicity of CTLs generated by stimulating splenic naïve T cells with fused NBT cells and fused NBT cells armed with GP115xCD28 BiMab *in vitro*.**

Effector cells	E:T ratio (1:20)			
	NBT-II	BERH-2	C6	YAC-1
CTLs (Fused NBT-B)	35%	8%	10%	7%
20 CTLs (Fused NBT-D)	33%	10%	11%	8%
CTLs (Fused NBT-BxBiMab)	67%	12%	12%	10%
<u>CTLs (Fused NBT-DxBiMab)</u>	<u>55%</u>	<u>10%</u>	<u>13%</u>	<u>11%</u>

	SMCC-1	Hepa 1-6	P815	YAC-1
25 CTLs (Fused SMCC-1-B)	28%	5%	6%	4%
CTLs (Fused SMCC-1-D)	21%	6%	5%	5%
CTLs (Fused SMCC-1BxBiMab)	50%	8%	7%	7%
<u>CTLs (Fused SMCC-1DxBiMab)</u>	<u>44%</u>	<u>5%</u>	<u>8%</u>	<u>4%</u>

Table VI. Nylon wool-enriched naïve splenic T cells (5×10^6 /well) were first stimulated by incubation with 2×10^5 irradiated (5000 roentgens) fused tumor vaccine cells or fused tumor vaccine cells armed with GP115xCD28 BiMab *in vitro*. Cytotoxicity of CTLs toward syngeneic, allogenic tumor cells and a NK sensitive YAC-1 cell line was determined in a standard 4 hour ^{51}Cr releasing assay. Results from three experiments are shown as the percentage of ^{51}Cr release as measure of tumor cell lysis.

The fused tumor vaccine cells armed with anti-CD28 Bi-MAb were also investigated for their effectiveness in eliciting preventive and curative immunity.

For prevention experiments, groups of C57BL/6 mice were immunized subcutaneously with 1×10^6 fused tumor cells armed with Bi-MAbs or with control cells. After two weeks, mice were challenged with 2×10^6 parental tumor cells (Table VII).

For curative experiments, mice were first injected subcutaneously with parental tumor cells. After two or four weeks, mice bearing tumors were then treated by subcutaneous injection with either γ -irradiated or non-irradiated fused tumor cells armed with Bi-MAbs or with control Bi-MAb. All animals were monitored weekly and tumor size was measured using a caliper. Tumor tissues from the treated mice were routinely subjected to histopathology examination for determination of local immune responses and phenotypes of tumor infiltrating T lymphocytes as described previously

Table VII			
<u>Immunization</u>	<u>Injecting cell No.</u>	<u>Challenge cell No.</u>	<u>Animals with tumor</u>
BCF-hepa 1-6	1×10^6	2×10^6	0/5
	1×10^5	2×10^6	2/5
	1×10^4	2×10^6	5/5
DCF-hepa 1-6	1×10^6	2×10^6	0/5
	1×10^5	2×10^6	3/5
	1×10^4	2×10^6	5/5
BCF-hepa 1-6-Bi-MAb	1×10^6	2×10^6	0/5
	1×10^5	2×10^6	0/5
	1×10^4	2×10^6	2/5

DCF-hepa 1-6--Bi-MAb	1x10 ⁶	2x10 ⁶	0/5
	1x10 ⁵	2x10 ⁶	1/5
	1x10 ⁴	2x10 ⁶	3/5
BCF-SMCC-1	1x10 ⁶	2x10 ⁶	0/6
5	1x10 ⁵	2x10 ⁶	2/6
	1x10 ⁴	2x10 ⁶	6/6
DCF-SMCC-1	1x10 ⁶	2x10 ⁶	1/6
	1x10 ⁵	2x10 ⁶	3/6
	1x10 ⁴	2x10 ⁶	6/6
BCF-SMCC-1 -Bi-MAb	1x10 ⁶	2x10 ⁶	0/6
10	1x10 ⁵	2x10 ⁶	0/6
	1x10 ⁴	2x10 ⁶	3/6
DCF-SMCC-1-Bi-MAb	1x10 ⁶	2x10 ⁶	0/6
	1x10 ⁵	2x10 ⁶	1/6
15	1x10 ⁴	2x10 ⁶	3/6

EXAMPLE 12

PHYSICAL ATTACHMENT OF Bi-MAb TO TUMOR CELLS BEFORE THEIR CONTACT WITH T CELLS IS MORE EFFECTIVE IN GENERATING CTLs THAN

PROVIDING Bi-MAb IN CELL CULTURE MEDIUM

- 20 Proliferation of CD3⁺CD8⁺CD25⁺ T cell population was determined and compared cytotoxicity of CTLs generated by contacting naïve T cells with (a) cytokine-treated tumor cells in the presence of soluble Bi-MAb in culture medium or (b) cytokine-treated tumor cells armed with GP115xCD28 Bi-MAb without adding soluble Bi-MAb to the culture medium.
- 25 Both cytokine-treated mouse and human tumor cells armed with GP115xCD28 Bi-MAb are effective in stimulating T cell proliferation and generating tumor specific CTLs *in vitro*. In contrast, addition of antiCD28 Bi-MAb into cell culture is much less effective in stimulating

T cell proliferation and generating CTLs (Table VIII). The data clearly showed that, to create strongly immunogenic tumor cell vaccine, it is very important to arm cytokine-treated tumor cells with anti-CD28 Bi-MAb *in vitro* before the contact between T cells and the cellular vaccine.

5 Without being bound by any theory, applicant proposes that the addition of soluble Bi-MAb to cell culture is less effective because a significant proportion of the soluble Bi-MAb will bind to T cells or tumor celis but not to both. For example, a CD28 on T cell surface bound by a Bi-MAb will not be accessible to another Bi-MAb bound to the tumor cell. Such conflict between Bi-MAb bound to CD28 on one hand and Bi-MAb bound to the 10 tumor cell on the other hand reduces the linkage between T cells and the tumor vaccine cells and thus the effectiveness of the tumor vaccine in stimulating T cell proliferation and generating CTLs.

15 **Table VIII. CD3⁺CD8⁺CD25⁺ T cell proliferation and cytotoxicity of CTLs induced by stimulating naïve T cells *in vitro* with tumor vaccine cells armed with Bi-MAb or tumor vaccine cells in the presence of soluble Bi-MAb in culture medium.**

<u>Stimulators</u>	<u>CD3⁺CD8⁺CD25⁺ T cell ratio</u>	<u>Cytotoxicity(1:20)</u>
CT-hepa 1-6 armed with CD28Bi-MAb	78%	64%
CT-hepa1-6 with CD28Bi-MAb in medium	37%	26%
20 CT-hepa 1-6 armed with CD18Bi-MAb	14%	9%
<u>CT-hepa1-6 with CD18Bi-MAb in medium</u>	<u>11%</u>	<u>7%</u>
CT-SMCC-1 armed with CD28 Bi-MAb	68%	71%
CT-SMCC-1 with CD28Bi-MAb in medium	31%	29%
25 CT-SMCC-1 armed with CD18 Bi-MAb	16%	12%
<u>CT-SMCC-1 with CD18 Bi-MAb in medium</u>	<u>12%</u>	<u>10%</u>
CT-SL-22 armed with CD28 Bi-MAb	54%	47%

CT-SL-22 with CD28 Bi-MAb in medium	10%	17%
CT-SL-22 armed with CD18 Bi-MAb	6%	7%
<u>CT-SL-22 with CD18 Bi-MAb in medium</u>	<u>5%</u>	<u>6%</u>

5

EXAMPLE 13**ADOPTIVE IMMUNOTHERAPY FOR XENOGRAFTED HUMAN
HEPATOCELLULAR CARCINOMA IN SCID MICE**

CTLs generated by stimulating T cells with cytokine treated hepa1-6 cells armed with anti-CD28 Bi-MAb are effective in eliminating established tumors in syngeneic animals when administrated intravenously at a dose of $2 \times 10^7 \times 3$ in combination with I.P. injection of IL-2 (1,000u \times 10). This approach is also effective in curing human xenografted tumors in immuno-deficient mouse. Three human hepatocellular carcinoma cell lines have been established from primary liver cancer tissues. All three hepatocellular carcinoma cell lines express GP115 antigens on their cell surfaces and have similar tumor formation rate to develop subcutaneous tumors when injected into nude or scid mice.

Peripheral blood T cells (PBTs) and tumor infiltrating T lymphocytes (TILs) were obtained from individual patients using a standard procedure. Briefly, T lymphocytes were obtained by stimulating these T cells with antihuman-CD3 Mab (1ug/ml) in the presence of 100ug/ml recombinant human IL-2. The T cells were stimulated periodically with antihuman CD3 monoclonal antibody (1ug/ml). Irradiated peripheral blood lymphocytes from normal donors were used as feeder cells. TILs were generated from fresh tumor tissue by culturing tumor tissues in recombinant-human IL-2 medium (100u/ml). Approximately, 55-75% of T cells and TILs were CD3 $^+$ CD8 $^+$ CD25 $^+$ T cells as tested by immuno-fluorescent analysis. Neither T cells from peripheral blood nor TILs showed cytotoxic activity toward autologous tumor cells *in vitro* as assessed by a standard 4 hour ^{51}Cr releasing assay.

The cytokine treated HCC cells armed with gp115:anti-CD28 Bi-MAb were used to stimulate autologous PBTs or TILs for the generation of CTLs (CD8 $^+$ T cell clones) for evaluation in a human xenografted HCC system.

In preventive experiments, groups of scid mice were injected i.v. with 1×10^7 CTLs or

control T cells and received, starting on day 2, i.p. injection of 1,000 u of r-IL2 every day for 7 days. Seven days after CTLs infusion, the mice were injected s.c. with 2.5×10^6 autologous parental tumor cells. The tumor growth was monitored daily in each group (Table IXa).

5 In curative experiments, scid mice were first injected s.c. with parental tumor cells at a dose of 5×10^6 cells) into right back of mouse. Two weeks later, mice with 0.5×0.5 cm tumor were injected twice i.v. with 5×10^7 CTLs or control T cells, followed by i.p. injection of 1,000u r-IL-2 every day for 7 days. Tumor regression and survival time were observed and recorded (Table IXb).

10 **Table IX. Therapeutic efficacy of adoptive transfer CTLs generated by stimulating T cells *in vitro* with cytokine treated tumor cells armed with anti-CD28 monoclonal antibody.**

Table IXa

	<u>CTLs/Stimulators</u>	<u>CTL Dose</u>	<u>Tumor formation</u>
15	CT-SL22 armed with anti-CD28Bi-MAb	1×10^7 (x2)	1/10
	CT-SL22 armed with Control Bi-MAb	1×10^7 (x2)	9/10
	<u>SL22 armed with anti-CD28 Bi-MAb</u>	<u>1×10^7 (x2)</u>	<u>10/10</u>

Table IXb

	<u>CTLs/Stimulators</u>	<u>CTL Dose</u>	<u>Tumor regression</u>
20	CT-SL22 armed with anti-CD28Bi-MAb	5×10^7 (x2)	7/10
	CT-SL22 armed with Control Bi-MAb	5×10^7 (x2)	0/10
	<u>SL22 armed with anti-CD28 Bi-MAb</u>	<u>5×10^7 (x2)</u>	<u>0/10</u>

EXAMPLE 14

ERADICATION OF TUMOR BY INTRATUMOR INJECTION OF CYTOKINES
AND Bi-MAb

A method developed to avoid this toxicity is intralesional administration of

5 immunotherapy, for instance by injection directly into the tumor or other diseased cells. Intralesional administration does not cause the toxicity seen with systemic administration of immunologic agents. Intralesional administration is also preferred when the lesion to be treated is not well vascularized, is inaccessible for biopsy, or cannot be disrupted without creating further risk to the patient. In a preferred embodiment, fine needle injection (FNI) 10 techniques are used to provide cytokines and bridge molecules of this invention to the lesion site. For example, the techniques described and cited in Chang et al., "Phase I clinical trial of allogeneic lymphocyte culture (cytoimplant) delivered by endoscopic ultrasound (EUS)-guided *fine needle injection* (FNI) in patients with advanced pancreatic carcinoma" Castroenterology (1997) 112(4):A546 can be used for this invention. The injection can be 15 assisted by imaging systems that allow physicians to visualize locations of lesions and providing the therapeutics to the right locations. The injected cytokines and Bi-MAbs are allowed to come into direct contact with tumor cells and generate CTLs targeting the tumor cells *in vivo*. In a preferred embodiment, the Bi-MAbs or other bridge molecules bind to an antigen present on the target diseased cells but not present on normal tissues at the disease 20 location so as to avoid generating immune response against healthy cells. In another preferred embodiment, the Bi-MAbs or other bridge molecules bind to an antigen unique to the target diseased cells.

Cytokines and Bi-MAbs can be injected together or separately. In one embodiment, cytokines are injected into lesion locations to increase the level of one or more primary and 25 costimulatory T cell activation molecules in target diseased cells before Bi-MAbs or other bridge molecules are injected to the same location. In another embodiment, cytokines and bridge molecules are mixed and injected to the lesion locations. Nucleic acids encoding and capable of expressing cytokines are injected to the lesion location in lieu of cytokines.

Unit dosages of cytokines and Bi-MAbs can be provided in separate containers or in 30 the same container. In that regard, the present invention features a sterile vial or other

container holding a composition comprising a unit dosage of the cytokines, Bi-MAbs, or the two in combination. Typically, the vial or container will bear a label setting forth information concerning the pharmaceutical use of the composition in treating a tumor (or other diseases) in a human, such as FDA approval for use of the composition in the treatment of a human

5 having one or more of the tumors (or other diseases) against which the method of treatment of the invention is effective as described herein.

Other molecules capable of increasing the level of one or more primary and costimulatory T cell activation molecules in target diseased cells may also be provided in lieu of or in addition to cytokines, e.g., MHC and B7.

10 Human patients with localized and surgically accessible tumors are logical candidates for intralesional immunotherapy. Both primary tumors and secondary or metastatic tumors can be treated in this way. Examples of tumors that can be treated by the method of this invention include the following:

15 Brain tumors, such as glioma, astrocytoma, oligodendrolioma, ependymoma, medulloblastomas, and PNET (Primitive Neural Ectodermal Tumor);

Pancreatic tumors, such as pancreatic ductal adenocarcinomas;

Lung tumors, such as small and large cell adenocarcinomas, squamous cell carcinoma, and bronchoalveolar carcinoma;

20 Colon tumors, such as epithelial adenocarcinoma, and liver metastases of these tumors;

Liver tumors, such as hepatoma, and cholangiocarcinoma;

Breast tumors, such as ductal and lobular adenocarcinoma;

Gynecologic tumors, such as squamous and adenocarcinoma of the uterine cervix, and uterine and ovarian epithelial adenocarcinoma;

25 Prostate tumors, such as prostatic adenocarcinoma;

Bladder tumors, such as transitional, squamous cell carcinoma;

Skin tumors, such as malignant melanoma; and

Soft tissue tumors, such as soft tissue sarcoma and leiomyosarcoma.

30 The results above have shown that effective tumor vaccines can be generated by treatment of tumor cells with a combination of cytokine and pre-incubated with an anti-CD28

Bi-MAb. This two-step process can be effectively modified by intratumor injection of a combination of cytokine and an anti-CD28 Mab (or other bridge molecules) which has a binding site to recognize antigens specific expressed on mouse cancer cells such as gp210, gp115 and gp55. The efficacy of intratumor injection of cytokine and Bi-MAb in induction 5 of anti-tumor immunity was first determined in syngeneic hepa 1-6 and SMCC-1 mouse tumor model.

Groups of C57 mice were subcutaneously injected with 1×10^6 hepa 1-6-tumor cells. Two weeks after tumor inoculation, mice with grossly identifiable tumors were injected 10 intratumorally with a combination of 200 u IFN- γ and 200u TNF- α daily x 3 and followed by an intratumor injection of Bi-MAb at a dose of 500ug at the 3rd day after last injection of cytokines. Mice taken intratumor injection of both cytokines and Bi-MAb showed significant tumor regression and extended survival time. This experiment supports that intratumor injection of cytokines and Bi-MAb is a way of generating tumor vaccines *in vivo*.

15

Table X

	<u>Tumor models</u>	<u>Cytokines</u>	<u>Bi-MAb</u>	<u>Tumor regression</u>	
	Hepa 1-6	IFN- γ +TNF- α		0/6	0/4
	Hepa 1-6		GP115xCD28	0/6	0/4
	Hepa 1-6	IFN- γ +TNF- α	GP115xCD28	4/6	3/4
20	SMCC-1	IFN- γ +TNF- α		0/5	0/5
	SMCC-1		GP115xCD28	0/5	0/5
	SMCC-1	IFN- γ +TNF- α	GP115xCD28	3/5	4/5

EXAMPLE 15

25

INTRA-SPENIC ADMINISTRATION OF VACCINES

It is likely that the initial contact between T cells and vaccine occurs within peripheral lymph organs such as spleen or lymph nodes rather then at the site of primary injection.

Injection to a peripheral tissue site (such as skin) or intravenously may lead to a substantial loss of DCs during migration into spleen or lymph node. Therefore, direct injection into a lymphoid organ may be desirable. Our data showed that a single intrasplenic injection of 2.5 x10⁴ cytokine treated hepa 1-6 cells armed with Bi-MAb was sufficient to elicit anti-tumor immune response against parental tumor cell challenge. Xenografted human melanoma and hepatoma cancer models can be used to determine the effectiveness of this approach and the minimum number of cytokine treated tumor cells armed with Bi-MAb required for effective vaccination. Mice having reconstituted human immune system will be first inoculated subcutaneously with 2 x10⁶ human melanoma or hepatoma cancer cell lines. After either two or four weeks, mice bearing tumor will be injected intrasplenically with 1x10³, 1x10⁴, 2x10⁴, 5x10⁴ or 1x10⁶ cytokine treated autologous tumor cells armed with Bi-MAb or control Bi-MAb. Vaccine cells can be directly injected into spleen using a #31 needle via a surgical approach through the back. The clinical application of this approach can be extended to intra-lymph node injection in human.

15

EXAMPLE 16**HUMAN CLINICAL DATA****IMMUNOTHERAPY WITH CANCER VACCINE**

The above data show that tumor cells treated with a combination of cytokines and 20 armed *in vitro* with Bi-MAb became more immunogenic and can cure established hepatoma and colon carcinoma in both mouse and rat models. To evaluate clinical effectiveness of the cellular vaccines on treatment of human hepatocellular carcinoma (HCC) and colon cancer, applicant generated human cellular vaccines using this two-step process and treated 13 HCC patients and 5 colon cancer patients.

25 In HCC patients, stages were II in 3, III in 7, and IV in 3 and in colon cancer patients, all had distant metastasis. All treated patients had a pathological diagnosis.

Each patient received subcutaneous injection of 1-1.5 x 10⁷ tumor vaccines twice within a two week period. Fifteen patients experienced a 37.8-38.6 °C fever after the second injection that continued for 8 to 15 hours. No other toxic and allergic reactions were 30 observed. DTH reactions to autologous tumor cells were significantly increased in 11 of 18

patients. Two patients had complete regression of metastatic lesions and four patients had partial regression of metastasis after treatment. Primary tumor regression >30% in size occurred in four HCC patients. In two patients with significantly tumor regression, tumors became operable and were successfully excised. Histopathology demonstrated marked 5 necrosis and abundant lymphocyte infiltration in tumor tissues.

COMBINATION IMMUNOTHERAPY WITH CANCER VACCINE AND ADOPTIVE TRANSFER OF TUMOR SPECIFIC CTLs

11 hepatocellular carcinoma (HCC) and 5 gastric cancer (GC) patients were treated by 10 the combined immunotherapy. In HCC patients, stages were III in 5 and stage IV in 6. All GC patients had recurrent metastasis. Each patient was injected s.c. with $1-1.5 \times 10^7$ tumor vaccines twice within a two-week period followed by infusion of $1.7-2.1 \times 10^9$ TS-CTLs. All patients experienced $37.8-38.8^\circ\text{C}$ fever after the second injection of vaccine cells that continued for 8 to 20 hours. No other toxic and allergic reactions were observed.

15 DTH reactions to autologous tumor cells were observed in 13 of 17 patients. Proliferation of T cells from the treated patients (14 cases) was significantly increased. Four patients had a significant regression of metastatic lesions and three patients had a partial regression of metastasis. Ascites completely disappeared in two GC patients after treatment. Abundant lymphocyte infiltration and necrosis were demonstrated in tumor tissues from six 20 treated patients. The results suggest that the combination of cellular vaccines and adoptive TS-CTLs immunotherapy provide an improved immunotherapy strategy for treating human cancers.

PROTOCOLS

25 Cytokines treatment of the tumor cells: Cells were plated into 24-well tissue culture plates at concentration of 2×10^6 cells/ml in RPMI-1640 complete medium with IFN- γ (100 U/ml) and TNF- α (50 U/ml) added freshly. Incubate the plate in 5% CO₂, 37°C incubator for 48 h.

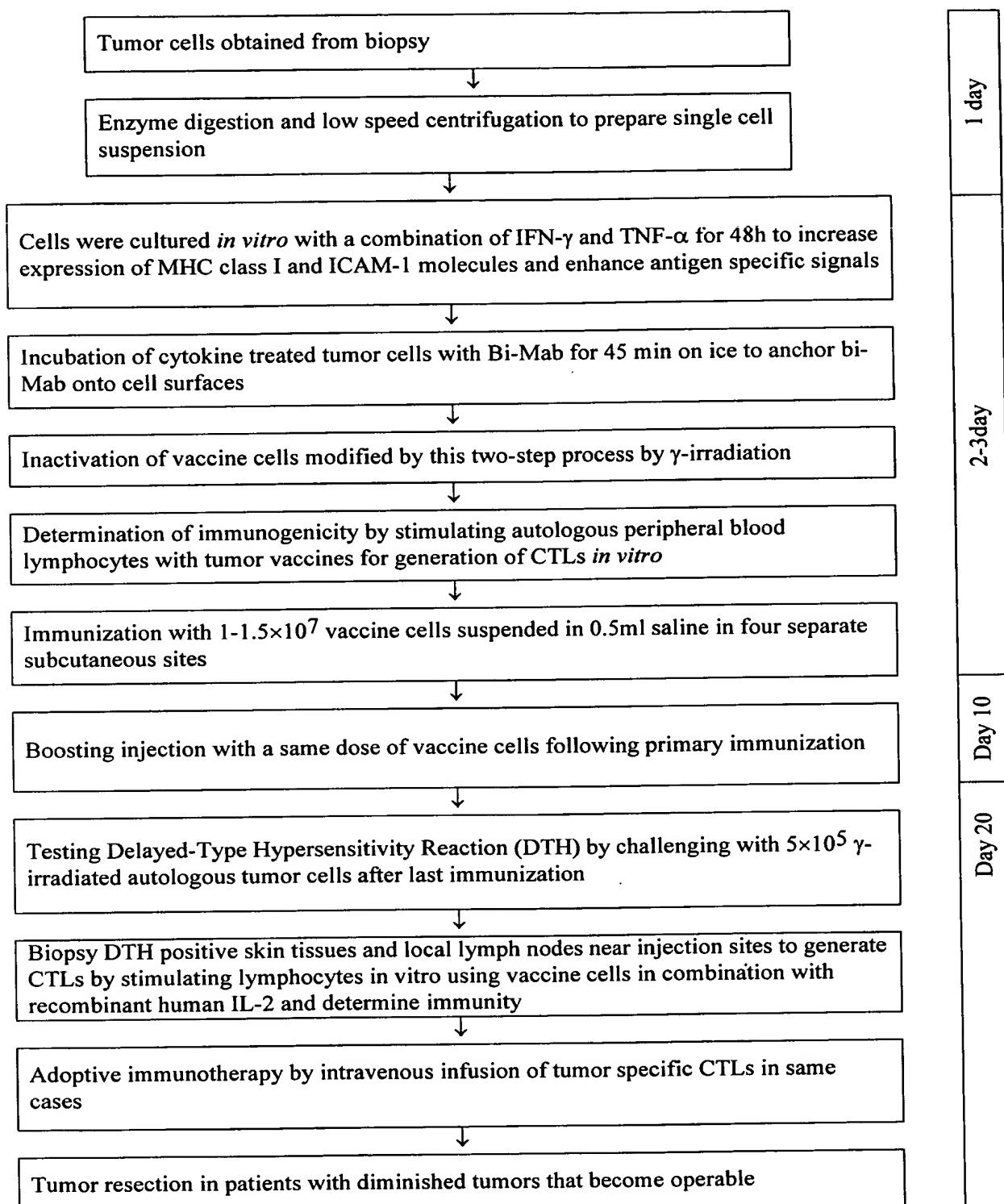
30 Preparation of cellular tumor vaccines in vitro: After treated for 48 h in vitro with a combination of IFN- γ and TNF- α , cells were then washed with PBS (pH 7.4) \times 3 at room temperature and incubated with anti-CD28 Bi-MAb at a concentration of 50 $\mu\text{g}/\text{ml}$ on ice for

45 min. After being washed, cells were subjected to an additional incubation in an equal volume of 30% polyethylene (PEG) in RPMI-1640 at 4°C for 30 min as previously reported (Huang, et al., (1994) Science 264:961-5). Finally, the cells were again washed with PBS \times 3 and suspended in a final concentration of 1-2 \times 10⁷/ml PBS.

5 Generation of CTLs and cytotoxicity assays: To stimulate tumor-specific CTL responses purified peripheral blood T cells or TILs were primed with γ -irradiated (5000 rad) cytokine-treated tumor cells armed with Bi-Mab for 9 days in complete RPMI-1640 medium, supplemented with 20 units/ml IL-2. The cytotoxic activity of in vitro stimulated T cells was determined using the ⁵¹Cr release assay.

REF ID: A657260

Table XI



EXAMPLE 17

GENERATION OF HUMAN MELANOMA VACCINES

Eight human melanoma cell lines have been established from primary melanoma tissues of patients. These melanoma cell lines express gp115 antigens on cell surfaces and 5 develop subcutaneous tumors when injected into nude or SCID mice. Neither peripheral blood T cells (PBTs) nor tumor infiltrating lymphocytes (TILs) obtained from these patients showed any cytotoxic activity toward autologous tumor cells *in vitro* as assessed by a standard 4h ⁵¹Cr release assay.

The cytokine treated human melanoma cells armed with gp115:anti-CD28 Bi-MAb 10 will be tested for their ability to stimulate autologous PBTs or TILs for the generation of CTLs. CD8⁺ T cell clones will be established by the stimulation of cytokine treated human melanoma cells armed with gp115:anti-CD28 Bi-MAb. The cytotoxic specificities of the CD8⁺ T cell clones toward various human tumor cells will be evaluated in a human xenografted melanoma system.

15 Before human testing, it will be determined whether adoptive transfer of these CD8⁺ T cell clones into SCID mice bearing autologous human melanomas can inhibit tumor growth and cure the established tumors. For example, SCID mice, in groups of five, will be injected subcutaneously with human melanoma cells in the right flank of mice (5×10^6 /each). Two weeks after tumor inoculation, mice with grossly identifiable tumors will be injected 20 intravenously with 2×10^7 CD8⁺ T cells and followed by a daily administration of 1,000u recombinant-human IL-2 $\times 10$. Tumor regression and survival time will be monitored. If tumor regression occurs, some of the mice will be sacrificed and tumor tissues will be examined by histopathological approaches. Different numbers of CD8⁺ T cell clones will be 25 adoptively transferred to allow the comparison of the effectiveness of the T cell clones in eradicating the established tumors.

The present invention is not to be limited in scope by the embodiments disclosed herein, which are intended as single illustrations of individual aspects of the invention, and any which are functionally equivalent are within the scope of the invention. Various modifications of the invention, in addition to those shown and described herein, will become 30 apparent to those skilled in the art from the foregoing description, and are similarly intended

to fall within the scope of the invention. For example, Guo et al., Nature Medicine, vol. 4:451-455, (April, 1997) provide examples and references.

5 All publications referenced are incorporated by reference herein, including drawings, nucleic acid sequences and amino acid sequences listed in each publications. All the compounds disclosed and referred to in the publications mentioned above are incorporated by reference herein, including those compounds disclosed and referred to in articles cited by the publications mentioned above.

Other embodiments of this invention are disclosed in the following claims.

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